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CONTENTS.

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No. B 781—June 1, 1933.

No. B 782—July 1, 1933.

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Section B.—Biological Sciences.

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Experimental Researches on Vegetable Assimilation and Respiration. XXI.—Induction Phases in Photosynthesis and their Bearing on the Mechanism of the Process.*

By G. E. Briggs, St. John's College, Cambridge.

(Communicated by F. F. Blackman, F.R.S.—Received January 13, 1932.)

In many reactions proceeding in living cells the concentration of the reactants changes with time, and hence our knowledge of the nature of the mechanism cannot proceed very far without means of determining, directly or indirectly, the changes of concentration. In photosynthesis the reactants carbon dioxide and water can often be readily maintained at a constant level, and consequently investigations have been centred on the relation between the rate of photosynthesis, when it has reached a stationary value, and the concentration of the reactants and various inhibitors and accelerators. Transitions from one stationary state to another, consequent upon a change in intensity of one of the determining factors, have received little attention. Investigation of such transitions may throw light on the mechanism of the process itself, and possibly on the interaction of this and other processes in the cell. The transition to be considered in this paper is that taking place in the rate of photosynthesis when chlorophyll-containing cells pass from darkness to

* This series of investigations carried out in the Cambridge Botany School was started by Dr. F. F. Blackman in 1895. Parts I, II and III appeared in 'Phil. Trans.' B, vols. 186, 186 and 197; Parts IV to XX, except the undermentioned in 'Proc. Roy. Soc.', B, vols. 76, 82, 82, 83, 83, 91, 92, 94, 94, 102, 102, 103 and 105; Parts VII, X, XI and XII in 'Ann. Bot.', vols. 24, 25, 27 and 33.

illumination. Perhaps, in view of the interactions between photosynthesis and respiration which we are going to suggest, as well as for other reasons, we ought to say net oxygen production or carbon dioxide consumption rather than photosynthesis.

In 1918 Osterhout and Haas (1918) gave the data for one experiment on *Ulva* in sea water exposed to bright sunlight which, according to the authors, showed "that *Ulva* which has been kept in the dark begins photosynthesis as soon as it is exposed to light, and that the rate steadily increases until a constant speed is attained." Actually their experiments tell us nothing about the rate in the earliest stages; only, that in the first 35·7 minutes there was as great a change in the concentration of hydrogen ions of the sea water as was eventually achieved in 20·4 minutes. The authors developed two theories to explain their set of data.

Warburg later (1920) carried out experiments with *Chlorella* at 25° in carbonate-bicarbonate mixtures giving relatively high concentrations of free carbon dioxide. Some experiments were done at high intensities of illumination and some at low. In only one experiment, at high illumination, was the change of rate subsequent to illumination followed. The other experiments suggest that when the illumination is weak the average rate for the first minute of illumination is essentially the same as that after the first minute.

The data of the above experiments and those of other workers will be considered in detail in the light of the theoretical schema we shall put forward after giving an account of our own results.

In addition to the above there are the results of experiments with rapid alternation of light and dark which may be used to give information on the changing rate of photosynthesis subsequent to darkening. These we propose to consider in detail on another occasion.

Experimental.

Technique.—The plant material used for most of the experiments was a moss, *Mnium undulatum.* Various leaves of Angiosperms gave similar results. The moss was used because it was little disturbed by the experimental conditions, recovered from accidental exposures to anærobic conditions, and could be used day after day for a week or more. Further, being devoid of stomata, the possible complication of change of stomatal aperture subsequent to illumination was avoided. The material was normally enclosed in a chamber containing 4 per cent. to 5 per cent. carbon dioxide diluted with hydrogen.

This concentration of carbon dioxide was high enough to ensure that the decrease during the experiment had no measurable effect on the rate of assimilation. The chamber was in series with a pump and a tube containing freshly dried palladium black. The pump changed the atmosphere of the chamber several times a minute. The oxygen evolved during illumination was equal to the carbon dioxide used up by the material. This equality was shown by the fact that the volume of the total gas underwent no change when the tube containing the palladium black was cut out of the circuit. The circulation of the gases through the palladium black resulted in a complete removal of the oxygen along with twice as much hydrogen. Hence the volume of the gases (reduced to N.T.P.) decreased three times as rapidly as the material changed carbon dioxide into oxygen. This apparatus, devised by Dr. F. F. Blackman, has been used in several other investigations of assimilation in this laboratory, some of which have been published (Briggs, 1929).

For the purpose of studying the induction period, viz., the phase which starts at the moment of illumination and continues as long as the rate continues to increase, the following procedure was adopted. Oxygen was either allowed to accumulate in the plant chamber by cutting it off from the rest of the apparatus during illumination, or else oxygen was introduced from outside. The plant chamber was then darkened for a time. Immediately before the commencement of the illumination the oxygen remaining in the chamber was removed by pumping the gas through the palladium tube. This removal occupied but a minute or two. The plant material was then illuminated and the change in the volume of the gases in the apparatus (reduced to N.T.P.) was followed. By the procedure mentioned previously it was shown that during the induction period the oxygen evolved was equal to the carbon dioxide consumed. When the palladium black was cut out of the circuit, there was a small increase of volume in the first few minutes. As we shall show later, this increase is caused by the temperature of the gases in the apparatus rising above that of the water bath, the temperature of which was used in reducing the volumes to N.T.P.

In the earlier experiments an electric lamp was used as a source of illumination, later a more constant light source was found in the form of incandescent gas mantles burning gas under constant pressure.

Results.

In fig. 1 the decrease in volume (N.T.P.) is plotted against time for a typical experiment. Inaccuracies of 0·01–0·02 c.c. may arise through limitations

in the accuracy of the records of temperature and barometric pressure. The first point to be noticed is that the volume increased 0·08 c.c. in the first 3 minutes and another 0·02 in the next 5 minutes before the decrease commenced. This initial increase was greater the greater the intensity of illumination. There are various lines of evidence indicating that this increase is caused by the temperature of the gases in the apparatus rising above that of the water bath. Such a rise is to be expected since the plant material absorbs a great deal of the radiation incident upon it. Only a small fraction of that absorbed is used in assimilation, the remainder heats the leaf and so the surrounding gases. The volume was such that an increase in temperature of

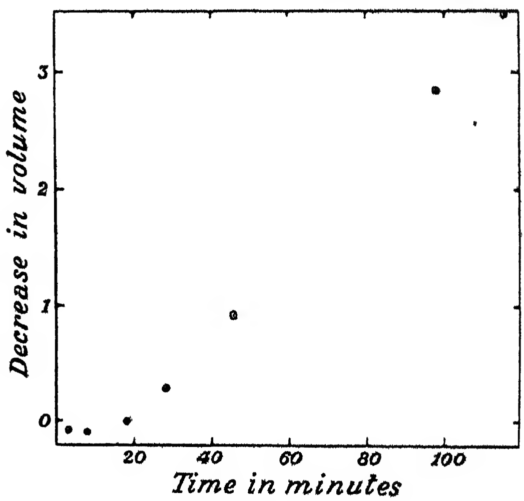

Fig. 1.

0·2° C. would cause an increase in volume of 0·1 c.c. When a thermocouple was inserted in a part of the apparatus shaded from direct illumination it was found that the difference between the temperature of the bath and that of the gases in the apparatus was of the order of 0·2° for an intensity of illumination of the magnitude of that used in the experiment recorded in fig. 1. This difference of temperature was attained within 10 minutes and did not increase subsequently. Similar increases in volume, using the bath temperature for reducing to N.T.P., were observed with dead material. When the chamber was darkened at the end of an experiment there was a contraction during the first 5 to 10 minutes of the same order or slightly bigger than the expansion on illumination; any change of volume owing to respiration during 10 minutes was too small to be recorded. Contractions of the same magnitude were observed with dead material and hence we can conclude that there was

practically no oxygen left in the apparatus at the moment of darkening after a period of illumination. An allowance for this preliminary expansion has been made on the basis of observations with dead material under appropriate conditions of illumination. In some experiments the expansion with the living material is less than that with the dead material, even during the first 3 minutes, indicating a measurable amount of oxygen production during this period.

From the curve shown in fig. 1 it would appear that there is a definite induction period during which the rate is increasing. As there was the possibility that the lag might be connected with the movements of the chloroplasts subsequent to illumination experiments were done with light filtered through a dense solution of potassium dichromate, which reduced the blue part of the spectrum to a negligible quantity. With such illumination the chloroplasts moved very little from their dark position during 2 hours, the duration of the assimilation experiments. Nevertheless a definite induction period was obtained with such illumination. Further, the fact that the lag tends to be most marked at high intensities of illumination, where change in the intensity has little effect on the rate of assimilation, and least marked at low intensities where change of intensity has a big effect on the rate, indicates that change in the intensity of illumination of the chloroplasts consequent upon moving from a shaded position to brighter position cannot play much part in causing the induction periods under discussion.

When an account of these experiments was given at the International Botanical Congress (1930) a suggestion was made (p. 421) that the change of rate might be caused by increase of the temperature of the plant material consequent upon the exposure to illumination. This clearly cannot be so. There was a marked change of rate under conditions of illumination where a change of temperature of 10° had little effect on the maximal rate of assimilation. Further, even if the initial rate was as high as the average rate for the first 10 minutes, the final rate was, sometimes, as high as fifty times the initial rate. With a doubling of the rate for each increment of 10° this would require an increase of the leaf temperature of 50°–60°. It was difficult to measure the temperature of the leaves of the moss. A thermocouple inserted in various angiospermous leaves under the brightest illumination never recorded a temperature of more than 1° above that of the gas in the apparatus. The thin moss leaves were probably much nearer to the gases in temperature. The thicker angiospermous leaves attained their excess temperature in about 10 to 20 minutes from exposure to the light; the thin moss leaves would be expected to attain a steady temperature more rapidly.

That the induction period is not due to the absence of oxygen from the atmosphere of the apparatus during illumination was shown by adding a known volume of oxygen immediately after the oxygen, remaining after the period of darkness, had been removed. The illumination was then started with the palladium black cut out of the circuit. After 10 or 20 minutes the total oxygen was absorbed by putting the palladium black into the circuit. The oxygen produced was obtained by subtracting the amount added at the outset. In all such experiments it was found that the average rate for the first 10 or 20 minutes in the presence of oxygen was less than the final rate. The difference was of the same order as when no oxygen was added at the outset.

With the apparatus used it was impossible to use concentrations of carbon dioxide other than those in which the rate of assimilation was practically independent of the concentration. Changes of concentration within this range (4 per cent. to 10 per cent.) had no discoverable effect on the induction period.* We restricted our investigations to a study of the relation between the induction period and intensity of illumination and temperature.

All our experiments recorded were carried out with a preliminary dark period of 2 hours or more. Periods shorter than an hour seemed to give a less marked induction, but the variability was so great that a very large number of experiments would be required to establish the relation between duration of darkness and magnitude of the induction period. In the experiments where the dark period was over 2 hours there is no significant correlation between the duration of the darkness and the lag. The one exception to the above statement is as follows. When the plant material was left for some time without oxygen there may be no oxygen production for a long time on illumination, perhaps an hour or more. This period of no oxygen production is longer the longer the period of exposure to anærobic conditions in the darkness. The moss was able to stand as much as 24 hours of anærobic conditions and eventually to assimilate after an hour or two of no oxygen production. Willstätter and Stoll (1918) record a similar experience with another moss, *Polytrichum*. To these experiments we shall refer later. Provided there was some oxygen left in the assimilation chamber there was no marked correlation between the amount left and the dimensions of the induction period.

* Subsequently Mr. H. G. Wager, by another technique has found that lowering the concentration to a point where the final rate is reduced has a similar effect on the induction phase to lowering the intensity of illumination, a point to which we shall return later.

Survey of the Effect of Conditions on the ratio of the Rate in the Early Stages to the Final Rate.

The experimental data consist of records of the amount of oxygen produced at intervals over a period of about 2 hours. During the early stages when the rate was changing more rapidly, observations were made at more frequent intervals than when the rate was approaching closely to its final value—in the early stages observations were usually made at 3, 6, 11, 18, and 28 minutes from zero time. Altogether 37 such records have been used. Many obtained with the electric lamp had to be rejected on account of the fluctuations in the intensity of illumination. All 37 have been used for figs. 2 and 3. A typical experiment is presented in fig. 1. Two records are given in detail in Table III. Several are presented in the form of the ratio of the rate to the final rate in figs. 4 and 5. Not all are included in these figures because in some experiments, especially those with weak illumination the ratios are so near unity, even in the early stages, that small experimental errors obscure the trend of the values plotted.

We shall have completely characterized an induction phase if we give the rate of oxygen production as a function of time, yet since it is impossible to use identical material in all the experiments there is much to be said for considering the ratio of the successive rates to the final rate rather than the rate itself. But it must be borne in mind that, although factors such as amount of material, will not affect this ratio directly, yet by causing differing degrees of self-shading of the material, the ratio may be affected indirectly. Further, other differences in the material, such as make-up of the assimilatory mechanism or amount of inhibitor, may affect the ratio directly. Despite these limitations a consideration of this ratio of rate to final rate as a function of time, under different external conditions, will be useful in an attempt at an analysis of the induction period. Moreover, these very differences in the induction period under the same experimental conditions can help in an analysis of the internal factors causing them.

In making a general survey of the data to ascertain what general form of schema will be needed to account for the facts the *ratio* for the rate in the very early stages is useful. When we have decided on the general form of the schema then it can be formulated more exactly to account, not only for the ratio in the early stages but also for the change in the ratio with time. Since the rate is changing continuously and only average rates for intervals can be obtained the final test will be a comparison of observed and calculated amounts of oxygen production.

The initial rate cannot be ascertained from the data with any high degree of accuracy, so we propose to consider the ratio of the average rate for the first 10 minutes to the final rate. Although the observations were not always made at 10 minutes from the start of illumination yet there are values on the progress curve quite close to this point, thus making interpolation easy. The ratios are plotted in figs. 2 and 3. The rate of assimilation is taken as the rate of oxygen production, no allowance being made for any oxygen consumed in

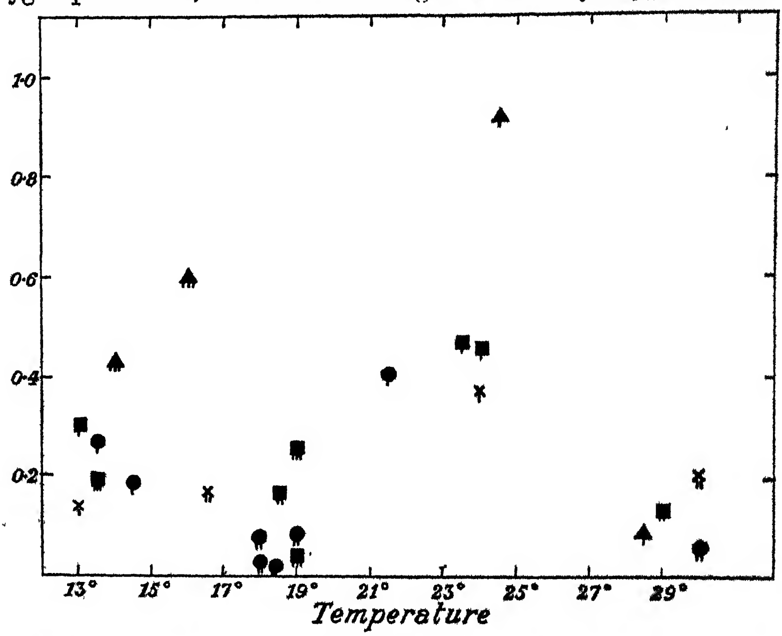

FIG. 2.—Ratio of average rate in first 10 minutes to final rate. (Gas burners.) × Lamp at 15 cm.; ● lamp at 20 cm.; ■ lamp at 28 cm.; ▲ lamp at 38 cm. I, Experiment series D; II, series E; III, series F.

respiration. We have data for oxygen consumption in the dark when the concentration of the oxygen was 4 per cent. to 5 per cent. Since the oxygen consumption by the cells, while assimilating in an atmosphere practically devoid of oxygen, is probably different from that in cells in the dark in an atmosphere containing oxygen, and in view of the possible interaction of assimilation and respiration, suggested later on, the addition of the respiratory consumption in the dark to the net production of oxygen in light is not likely to give us a more accurate figure for the assimilatory production.* An allowance for respiration based upon our measurements does not affect the generalizations

* On the whole (see Appendix) it seems probable that these values of apparent assimilation with very low oxygen concentration in the cells are a very close approximation to the values of real assimilation.

we shall make. For example, such an allowance generally increases the difference between the ratios for high and low intensities of illumination. The values for respiration would have to be many times greater, and yet greater for the high illumination, if these differences were to be obliterated. We shall therefore take the drift of the ratios of net oxygen production with intensity of illumination and temperature as being an indication of the drift of the ratios of assimilatory production.

An inspection of the figures will show that there is a very marked scatter of the points superimposed upon any drift with temperature or illumination. The scatter is not due entirely to variability with different samples of material. With one lot of material in successive experiments at the same intensity of

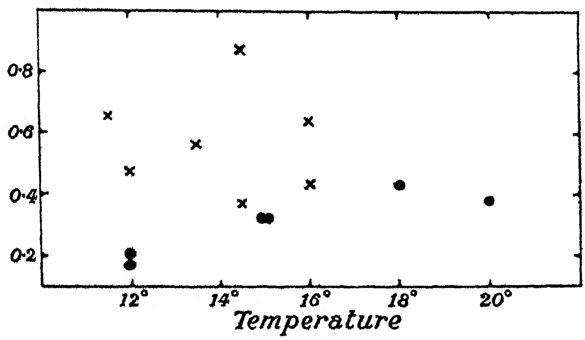

FIG. 3.—Ratio of average rate in first 10 minutes to final rate. (Electric lamp.) × Lamp at 45 cm.; ● lamp at 24 cm.

illumination, and temperatures that did not differ by more than 1°, the ratio was 0·09, 0·03, 0·08 and 0·02.

Underlying this scatter there will be seen in fig. 2 a very definite drift with temperature of the ratios for any one intensity of illumination. In fig. 3 constructed from experiments with an electric lamp a similar drift is suggested where the intensity of illumination was greater, if there is any drift with the weaker illumination it is largely obscured by the scatter. The experiments with the electric lamp are less reliable than those with gas mantles on account of fluctuations in the intensity of illumination during an experiment with the former source. The general indication is that the ratio tends to increase as the temperature is raised until about 25° C. is reached, and after that to decrease.*

* In fig. 2 the correlation coefficient for the ratios for the three higher intensities of illumination, even when all are taken together, is $r = 0 \cdot 39$. When the series E experiments are left out (for some reason these are clearly lower than the others), the correlation is increased and becomes more significant, $r = 0 \cdot 62$. With the higher illumination in fig. 3 the correlation with temperature is $r = 0 \cdot 66$. Only temperatures below 25° C. were used.

Secondly, it appears that the ratio for the lowest intensity of illumination is higher than that for the highest illumination; this comes out most clearly in fig. 3. The relative position for the intermediate intensities in fig. 2 is obscured by the scatter. Not a great deal of information can be given about the intensities of illumination from the point of view of assimilation, since the illumination of the material was far from uniform. In a mass of small leaves such as that used many were shaded by one or more other leaves. This much can be said; the highest intensity of illumination incident upon the chamber when the electric lamp was used was 8000–10,000 metre-candles (lux), and this at the lower temperatures approached the point where further increase of intensity had little effect on the final rate of assimilation. This was true despite the fact that some of the leaves were exposed to an illumination reduced by shading to a value less than that incident upon the other leaves.* The highest intensity with the gas burner was slightly less than that with the electric lamp, judging from the final rate of assimilation. The lowest intensity with the gas burner gave a final rate about a fifth to a sixth of that obtained with the highest intensity, when the temperature was about 15° C. Increase of temperature increased the final rate much more at the highest intensity than at the lowest; in the latter the effect was practically nothing. It was impossible to make at the same time, and with the same material, an accurate survey of the effect of temperature and of illumination on the rate of assimilation and upon the induction period. The activity of the material changed from day to day, and although the ratio of rate to final rate may be little affected by the amount of material, the final rate itself is much affected.

In comparing our results with those of Warburg (1920), who worked with two different intensities of illumination, it is to be noted that while he obtained a definite induction period with a high intensity there was practically none at the low intensity. Actually Warburg's induction period was much shorter than ours, the rate approaching very close to the final rate within a minute or so. He did experiments at one temperature only, namely, at 25° C., a temperature at which our initial rates approached most closely to the final rates.

With regard to the effect of light intensity on the induction period, Warburg's only comment is "Hätte die Induktion bei der Assimilation ähnliche Ursachen wie bei der Chlorknallgasreaktion, so müsste die Induktionszeit um so kürzer sein, je höher die Bestrahlungsintensität."

To face the problem fully we have to formulate a schema which will account

* Possibly the complication of shading is not much worse with a mass of thin moss leaves than with a thick angiospermous leaf.

for the following facts: after a period of darkness the rate of assimilation is relatively low and rises until a steady value is reached; the ratio of the rate in the very early stages to the final rate is greater with the lower intensity of illumination, and with higher temperatures between 11° and 25° C. We must also take into account the scatter of the ratios for a given set of external conditions and the possibility that the ratio falls when the temperature is increased above 25°.

We have worked out in detail many schemata, not only in relation to the above facts, but also in relation to the general body of knowledge of assimilation. There are obvious reasons for not considering all in detail, but it may be as well to examine some we consider less probable as well as that which in view of our present knowledge seems more probable.

First Schema.—The first schema to be considered is the one which we put forward when we gave an account of the data at the International Botanical Congress in 1930. The essential points of this schema are that there is in the assimilating cells a substance, S, which can be activated by light energy to a form S_l,* which then breaks down to reform unactivated free S substance, S_f, and the products of assimilation, and that alternative to activation the S substance may combine with an inhibitor, i, to form S_i. Further, it is assumed that the induction phase has nothing to do with the process of activation (the equilibrium between activated and unactivated S substance is assumed to be achieved very rapidly), but rather with the drift of compound S_i to its new equilibrium condition, consequent upon the change of the amount of S_f through the activation of some of this by radiant energy.

We have

$$S = S_l + S_i + S_f$$

In the dark

$$dS_i/dt = k_1 . S_f . i - k_2 . S_i.$$

When equilibrium is attained

$$S_i = S . i/(i + k_i) \quad \text{and} \quad S_f = S . k_i/(i + k_i).$$

The constant k_i is k_2/k_1. For simplicity we shall assume that the concentration of S relative to i is small so that the concentration of uncombined inhibitor

* The precise nature of the process need not be examined here. S may be chlorophyll, and S_l may be chlorophyll raised to a higher electronic level, or it may be dissociated chlorophyll. S may be a complex of chlorophyll and CO_2, or the activated chlorophyll may pass its energy on to CO_2 and the activated CO_2 may then give rise to the products of assimilation.

is the same as the total concentration. The rate at which S_i attains its equilibrium value depends upon the absolute values of k_1 plus k_2. This applies to the equilibration in the dark, and also when the dark equilibrium is disturbed by the introduction of a change in the situation on illumination. On illumination that part of the S substance now free rapidly comes to equilibrium in the new conditions. Such a rapid equilibration seems a necessary assumption to explain the phenomena of assimilation with rapidly intermittent illumination, the discussion of which we postpone till another time.

On illumination

$$dS_l/dt = k_3 . L . S_f - (k_4 + k_5)S_l \quad \text{and} \quad S_f + S_l = S_{f_0},$$

where S_{f_0} represents the dark equilibrium value of S_f, $k_4 . S_l$ the rate of breakdown of S_l to S_f with the liberation of the energy it had absorbed, $k_5 . S_l$ the breakdown to S_f and the products of assimilation, and L is a measure of the radiation. On account of the large value of $k_3L + k_4 + k_5$ this activation is assumed to attain equilibrium practically instantaneously. Hence at zero time

$$S_{l_0} = S_{f_0} . L/(L + k_l) = S . L . k_i/(L + k_l)(i + k_i).$$

The rate of assimilation is proportional to S_l.

On account of this sudden change in the amount of free S substance to a lower value S_i tends to a new equilibrium value. This as we have already stated is assumed to proceed relatively slowly. As S substance is liberated it instantaneously comes to equilibrium with the radiation. So the rate of liberation of free S from S_i determines the rate of formation of S_l and hence the rate of assimilation from moment to moment.

The rate of change of S_i is given by

$$dS_i/dt = k_1 . i . (S - S_i - S_l) - k_2S_i,$$

and at each moment the fraction $L/(L + k_l)$ of $(S - S_i)$ is in the active form S_l

$$S_l = (S - S_i) . L/(L + k_l)$$

From the above expressions the following relation between S_l and the time, t, can be derived in a straightforward manner

$$S_l = S_{l\infty} (1 - e^{-bt}) + S_{l_0} . e^{-bt}$$

where $S_{l\infty}$ the final value attained by S_l, is given by

$$S_{l\infty} = \frac{S . L}{L + k_l(1 + i/k_i)}$$

and

$$b = k_2 \, . \, \frac{L + k_l(1 + i/k_i)}{L + k_l} \, .$$

According to this schema the ratio of the initial rate to the final rate is given by

$$S_{l_0}/S_{l\infty} = \frac{L + k_l(1 + i/k_i)}{(L + k_l)(1 + i/k_i)}$$

and the ratio of the rate at any moment to the final rate by

$$S_l/\,S_{l\infty} = 1 - (1 - S_{l_0}/S_{l\infty})e^{-bt}.$$

An increase of intensity of illumination, L, leads to a decrease of $S_{l_0}/S_{l\infty}$ and, since b is also decreased, $S_l/S_{l\infty}$ also decreases. This is in agreement with our observation that the ratio of the average rate for the first 10 minutes to the final rate is less at the highest intensity of illumination than at the lowest.

With regard to the effect of change of temperature we must consider its effect on the values of the constants k_l and k_i and its possible effect on the amount of inhibitor, i. The former constant is the ratio of the velocity constant of a chemical to that of a photochemical reaction, and hence is most probably increased by increase of temperature. The constant k_i, on the other hand, is the ratio of the velocity constants of two chemical reactions and is most probably much less affected by temperature changes. Consequently as far as the effect of increase in temperature on these constants is concerned the effect on $S_{l_0}/S_{l\infty}$ and on b,* and hence on $S_l/S_{l\infty}$ should be the opposite of the effect of increase in intensity of illumination; that is the ratios should increase as the temperature is increased. Any increase in the concentration of inhibitor with rise of temperature, an eventuality to be taken into account if the inhibitor is a product of cell metabolism, will have an effect on the ratios which is opposed to the effect of the increase in k_l and k_2 which accompanies the increase of temperature. In this way our observed increase in the ratio of the average rate in the first 10 minutes to the final rate as the temperature increases from 11° to 25° can be accounted for by saying that the effect of the increase in temperature on k_l predominates over any effect consequent upon a tendency for the inhibitor to increase, and the fall of the ratio above 25° can be accounted for by an increase of inhibitor causing an effect greater than that consequent upon an increase of k_l and k_2.

* The effect of change of temperature on b is most marked, since this is proportional to k_2, a chemical velocity constant.

Lastly, if the inhibitor is a product of the cell metabolism it is not surprising that, even in the same material, it is different at different times, consequent upon different previous history. To such a variation in concentration of inhibitor we can attribute the fluctuations of the ratios for the same material under similar conditions at the time of experiment, and the general scatter of the points in figs. 2 and 3.

Having made this survey of the qualitative agreement of the facts with the expectations based upon the schema, it remains to test the agreement, not only for the first 10 minutes, but throughout the period of changing rate, and to make the comparison quantitative. We could obtain an idea as to whether there is a general agreement throughout the course of drifting rate by plotting the quantity $\log_e (1 - R/R_\infty)$ against time, where R is the rate of assimilation and R_∞ is the final rate. An inspection of the expression on p. 13 for S_l as a function of time will show that the above graph should be a straight line with slope b. To obtain values of the rate we should have to approximate by drawing tangents to the progress curves. Since we can readily obtain from our expression of rate as a function of time an expression for total assimilation at any time it is preferable to approach the question from this point.

We have

$$R = k_5 \,.\, S_l = k_5 \,.\, S_{l\infty} (1 - e^{-bt}) + k_5 S_l e^{-bt}.$$

Integrating between the limits t and 0 we have

$$A = R_\infty \left(t - \frac{1 - e^{-bt}}{b}\right) + R_0 \left(\frac{1 - e^{-bt}}{b}\right)$$

where A is the amount of assimilation at time t.

If A_1, A_2, and A_3 are the amounts of assimilation in successive intervals of time, of length T, we have

$$(R_\infty T - A_1)/(R_\infty T - A_2) = (R_\infty T - A_2)/(R_\infty T - A_3) = (e^{bT}).$$

This expression applies to net oxygen production as well as to "real assimilation" provided the respiratory consumption of oxygen is constant throughout the course of the experiment. In Table I are the values of assimilation in successive 10-minute intervals for three experiments. The differences as regard the induction period and the final rate for these experiments with the same material under similar conditions is attributed to different values for i, the concentration of inhibitor. From any three successive values for assimilation we can calculate the value for final rate, which will give the equality of the above expression, and compare it with the observed final rate. Using the

Table I.—Assimilation in successive 10-minute Intervals and Final Rate.

Experiment No.	Lamp distance.	Temperature.	Assimilation.				Final rate.		
			A_1 0-10.	A_2 10-20.	A_3 20-30.	A_4 30-40.	Calculated. A_1-A_3.	Calculated. A_2-A_4.	Observed.
	cm.	°C.							
E.II	20	19	0	13	31	35	−0·34	0·36	0·37
E.IV	20	18	1	22	42	46	2·21	0·47	0·48
E.III	20	18	9	31	43	49	0·57	0·55	0·53

values between 10 and 40 minutes it will be seen that the agreement is very good for all three experiments. Thus showing that the schema gives a good account of the facts from 10 minutes onwards. But when we use the values from zero time to 30 minutes we arrive at absurd values for the final rate in two of the experiments. To get the same values for the final rate using the figures from 0–30 as we do using those from 10–40 we should require the following values for the assimilation in the first 10 minutes: —68 for E.II, —78 for E.IV, and 7 for E.III. Thus it is seen that, except for the experiment where there is the smallest postulated amount of inhibitor (where the difference between initial and final rates is least) the theory fails to give an account of the progress during the first 10 minutes. In other words the theory fits only when the inhibitor is in relatively small amount. That the schema fails generally in this respect is shown by an inspection of fig. 4 where the average values of the quantity $\log (1 - R/R_\infty)$ are plotted against time for several experiments.* As we have already pointed out these values should fall on a straight line which cuts the axis when $t = 0$ at $\log (1 - R_0/R_\infty)$, that is below zero. In this case an allowance has been made for respiration on the basis of respiration in the dark. Since the change of rate during an interval is not a linear function of time we have used the average rate for an interval and drawn a line over the whole interval, rather than plot a point for half-way through the interval. Generally speaking the slope of the graphs increases at first. The experiment E.III is one where a straight line might be drawn through the data in the figure, but as we have already shown these data agree quite well with our schema. For other experiments very high values would have to be assumed for the respiration of the illuminated material if the data

* The later values, as R approaches R_∞, are affected more by experimental error than the earlier ones.

were to be made to fall on a straight line giving a value less than zero at $t = 0$. For example, in E.IV the rate of respiration in the dark was 0·3 (about one-sixteenth of the assimilation), but to make the first two points fall on a straight line with the rest a rate of respiration of 3·0 would have to be assumed for the interval 6–11 minutes, and one of 2·0 for the interval 11–18 minutes; values ten and seven times that of the respiration in the dark. These figures are based on the assumption that respiration falls to 0·3 when a steady rate of assimilation is reached, if it were higher then the above estimates would have

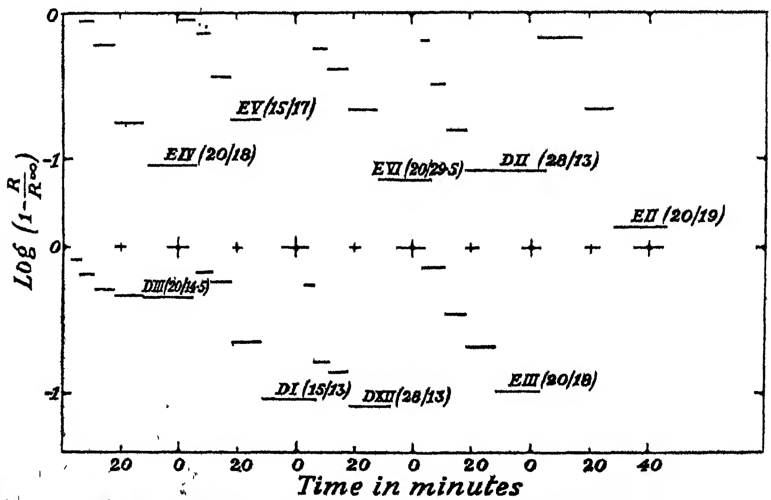

FIG. 4.—Log ($1 - R/R_\infty$) and time for nine experiments.

In figs. 4 and 5 the figures in brackets, *e.g.*, 20/18, mean lamp at 20 cm., temperature 18° C.; the other figures, *e.g.*, E.11, refer to the experiment number.

to be yet further increased. To bring all the data into agreement with the theory would need a fair amount of elasticity in the respiration value.

Another way of overcoming the misfit in the early period, or rather (in view of E.III) when the amount of inhibitor is relatively high, would be to postulate that each unit of the S substance has more than one centre which can be activated by radiant energy or alternatively combined with inhibitor, and that all centres must be activated by radiant energy before the unit can be effective in assimilation. This view has some attractions, since it appears that four or more quanta of visible radiation are necessary for the conversion of one molecule of carbon dioxide into the products of assimilation, and this as we have argued elsewhere may indicate a light-absorbing molecule built up of four or more distinct portions (Briggs, 1929). In such a picture, when the concentration of inhibitor is low few S units will have more than one centre

blocked by inhibitor, and hence our original schema would fit fairly well without modification. When the concentration of inhibitor is great there will be more S units with two centres or more blocked by inhibitor, and it will not be until all the centres but one of a unit have been vacated that our simple schema will apply. Until then the rate will be lower than expected.

As we increase the flexibility of our schema we require a greater and greater body of data to test its applicability. As the matter stands at present we can say that the above modification is in the right direction.

As far as we are aware there is no evidence in the field of photochemistry of an inhibitor of the type we have postulated, that is where the activation by light of a molecule is alternative to the reversible combination of that molecule with an inhibitor. This, in itself, is no sufficient reason for rejecting the theory; it might be an indication that the field of photochemical enquiry has so far been limited.* In any case alternative theories are worthy of consideration.

Depressants of the HCN *Type and the Mechanism of Photosynthesis.*—As we stated when discussing the first schema the evidence available seems to suggest that induction phases as long as those observed in our experiments are most probably due to some reaction secondary to those normally involved in the photosynthetic process. In our first schema this secondary reaction was the displacement of a purely hypothetical inhibitor from a part of the photosynthetic machinery, in our second it is the destruction of an inhibitor of the HCN type. This type of inhibitor causes a greater percentage depression with strong illumination than with weak (Warburg, 1920). If we assume that an inhibitor of this type is produced in our material under the experimental conditions, and that during assimilation it is directly or indirectly destroyed, then we have an explanation of a low initial rate of assimilation rising to a maximum when the inhibitor has been totally destroyed or has reached a lower stationary value than it had before illumination. Further, since the inhibitor is less potent at weak illumination we possibly have an explanation of the greater ratio of initial rate to final with weak illumination as compared with strong illumination. In this case we have an inhibitor the concentration of which depends upon a production, which goes on in the dark as well as in the light, and a consumption dependent upon the light. The final concentration obviously depends upon the intensity of illumination, and hence we have a

* If we adopt the view that chlorophyll is an optical sensitizer, absorbing radiant energy and passing it on to CO_2, the investigation of the effect of inhibitors on other processes of photosensitization would be of interest in this connection.

factor which tends to obscure the real relation between rate of assimilation and intensity of illumination.

We have considered in detail various possible forms of such a schema. They all involve a conversion of a substance, S, into an activated form by radiant energy (as in the previous schema); a breakdown of this by a substance, the effective amount of which is a function of the concentration of inhibitor; a production of the inhibitor by some metabolic process in the cell and a destruction consequent upon illumination.

Before we proceed with our problem of a precise formulation we must consider some of the facts concerning the effect of such inhibitors on the rate of assimilation. Various workers have suggested theoretical mechanisms for photosynthesis and for the mode of action of inhibitors, but so far little attempt has been made to test out in detail the agreement of fact and theory. As regards photosynthesis Willstätter and Stoll suggested that some complex of chlorophyll and carbon dioxide is converted by light into a peroxide which is broken down by an enzyme to give the products of assimilation and free chlorophyll. Warburg, in his first theory suggested that chlorophyll was converted by light into a "photochemical primary product," and that this reacted with a compound of CO_2 and a substance, B, the compound being the "acceptor." The formation of this acceptor he called the "Blackmansche Reaktion." Subsequently Warburg and Uyesugi (1924) adopted the view of Willstätter and Stoll, the breaking down of the peroxide becoming the Blackmansche Reaktion. We are not concerned here whether the experimental evidence produced justified the change of view.

In Warburg's theoretical discussions he appears to adopt the view as stated by Warburg and Uyesugi "Als Blackmansche Reaktion bezeichnen wir den chemischen Vorgang, der bei sehr starker Bestrahlung einer grünen Zelle das Tempo der Kohlensäurespaltung bestimmt." When Warburg comes to the point of formulating equations he is not consistent. For example, he says with regard to the formation of acceptor, if B is the total amount of cell substance and x the amount not combined with CO_2 then the rate of formation of acceptor is

$$v_1 = k_1 . x . c,$$

where c is the concentration of CO_2, and the rate of breakdown

$$v_2 = k_2 . (B - x),$$

k_1 and k_2 being chemical velocity constants. The quantity $B - x$ is obviously the amount of acceptor. Later in the same paper he says that the rate of

breakdown of the acceptor, C_A, when it reacts with the photochemical primary product is $k_3 . C_A . C_I$. Clearly then k_2 is not a chemical velocity constant but is equal to $k_3 . C_I$. He then proceeds to the equation

$$dC_A/dt = K - k_4 . C_A - k_3 . C_A . C_I,$$

where the second term represents the reversion of acceptor to free B and CO_2, which proceeds in the dark and the light, and the third term the reaction with photochemical primary product. Of the first term Warburg says the acceptor is formed at a constant velocity, K, in a constant concentration of carbon dioxide. This makes no allowance for his previous equation where the rate of formation of acceptor depends upon the amount of free B substance, (x), a quantity which depends upon its rate of reformation from C_A.

A consistent formulation of Warburg's first theory seems to be as follows. The rate of formation of photochemical primary product, S_l, equals

$$k_1 . L . (S - S_l),$$

where L is a measure of the intensity of illumination. The rate of destruction is $k_2 . S_l$ plus $k_3 . B_c . S_l$, where the first term represents the reversion to free S in ways other than those giving rise to products of assimilation and the second term the reaction with acceptor to give products of assimilation. The rate of formation of the acceptor, B_c, equals $k_4 . C . (B - B_c)$, where C represents the concentration of CO_2; the rate of destruction $k_5 . B_c$ plus $k_3 . B_c . S_l$, where the first term represents the spontaneous reversion. When a steady state is attained

$$k_1 . L(S - S_1) = S_l(k_2 + k_3 . B_c)$$

$$k_4 . C(B - B_c) = B_c (k_5 + k_3 . S_l).$$

As the value of L is increased the value of S_l approaches the limit S and the rate of assimilation approaches

$$k_3 . S . B_c = k_3 . k_4 . S . B . C/(k_4 . C + k_5 + k_3 . S).$$

The rate of formation of acceptor is obviously greater than this since some of the acceptor reverts spontaneously without reacting with the photochemical primary product. Clearly if this formation of acceptor is the Blackmansche Reaktion, it no more determines the rate of assimilation with high illumination than does the formation of primary product. Only if S is very great so that practically all the acceptor reacted with primary product and none reverted

could the Blackmansche Reaktion fix the rate of assimilation. Any factor altering S will change the rate also.

Warburg suggests that inhibitors such as HCN and H_2S depress the rate of assimilation by acting on the Blackmansche Reaktion.* Since he does not specify the precise mode of action, and in view of the above inconsistencies, we will continue our own formulation. When the concentration of CO_2 is high these substances depress more with strong illumination than with weak. According to the above formulation the rate of assimilation approaches the following value as the concentration of CO_2 is increased,

$$k_1 . k_3 . L . S . B/(k_1 . L + k_2 + k_3 . B).$$

If the effect of the depressants is to reduce the effective amount of B to the same extent whatever the intensity of illumination, then the percentage depression of assimilation will be greater the stronger the illumination. A simple formulation of the picture of Willstätter and Stoll would reduce to the same mathematical expression for high concentrations of carbon dioxide. We are not concerned now as to whether these simple formulations fit all the known facts of assimilation; we shall limit ourselves to the question of inhibitors.

According to both pictures the rate of assimilation at high illumination and high CO_2 is proportional to B, whether this is a compound of CO_2 with some cell substance or a catalyst breaking down a peroxide. Hence the relation between assimilation and concentration of inhibitor under these conditions gives us the relation between the effective amount of B and the concentration of inhibitor.

An examination of the relevant data renders it obvious that it is difficult to arrive at an exact statement of the relation. In most of the experiments of Warburg and his co-workers one reading for assimilation and one reading for respiration is recorded, and these are added together to give the value of real assimilation. In the paper by Warburg and Uyesugi data are given for 15 and 30 minutes. In many experiments the respiration in the second quarter hour is much less than that in the first. Taking three experiments without HCN, the two successive values were 26 and 8, 34 and 10, 8 and 8; with HCN present the values were 24 and 7, 35 and 15, 18 and 17. The apparent assimilation also fell; without HCN 43 and 40, 48 and 43, 48 and 39; with HCN 34 and 30, 18 and 16, —5 and 0. We are faced with the question as to

* There is no evidence as to the relative effect of H_2S at weak and strong illumination; we have only Negelein's (1925) assumption that this substance acts on the Blackmansche Reaktion.

which value we are to take for the apparent assimilation and which value of respiration we are to add to give the real assimilation. When the respiration is a small fraction of the assimilation it does not matter much provided the respiration of the illuminated cells is the same as that of the darkened.

If the HCN formed a simple reversible compound with the B substance, the compound being ineffective, then the effective amount of B would be

$$B \,.\, k_i/(i + k_i),$$

where i is the concentration of the inhibitor and k_i the dissociation constant of the compound with B. The relation between the rate of assimilation with high carbon dioxide and high illumination in the presence of inhibitor, R_i, and R, the rate in its absence, is $R/R_i - 1 = i/k_i$.

In Table II we give the figures from experiments by Warburg and Uyesugi, and by Warburg. The value for the real assimilation was obtained by adding the respiration of the darkened cells for the corresponding period. If our

Table II.—The Depressant Effect of HCN on the Assimilation of *Chlorella* at high concentration of CO_2 and strong illumination. Data from Warburg and Uyesugi, and Warburg.

Concentration of HCN.	Apparent assimilation.	$R/R_i - 1$.	Real assimilation.	$R/R_i - 1$.
0	43 83		47 89	
5×10^{-6} m.	34 64	0·264 0·297	39 71	0·204 0·252
0	48 91		52 97	
10^{-4} m.	18 34	1·66 1·68	23 43	1·26 1·26
0	48 97		52 103	
10^{-3} m.	−5 −5	Negative	2 4	25·0 24·8
0 10^{-3} m.	444 16	26·7	456 62	6·35
0 2×10^{-3} m.	480 4	119	492 50	8·8
0 5×10^{-3} m.	294 −2	Negative	306 27	10·4

formulation is correct the quantity $(R/R_i - 1)$ should increase proportionally with the amount of HCN added. It is seen that the increase is too slow in the dilute and stronger concentrations, and too rapid in the intermediate. As to what happens with the actual assimilation, not that obtained by adding the respiration to the apparent, we cannot say. Evidence of complications is revealed by the fact that the value of i/k_i is so different for different experiments with 10^{-3} M HCN.* In one place Warburg argues that HCN has a smaller depressant effect on the assimilation of the CO_2 of respiration than on the assimilation of CO_2 from outside the cell, but on all other occasions he takes the depression of real assimilation as a measure of the depressant effect on the Blackmansche Reaktion.

For the effect of H_2S Negelein's data (1925) are for apparent assimilation: 124 with no H_2S, 109 with 10^{-6} m., and 25 with 10^{-5} m. Using these values $(R/R_i - 1)$ is 0·135 and 2·52 respectively. Making allowances for respiration on the basis of other experiments (5 in the absence of H_2S, 6 with 10^{-6} m. and 9 with 10^{-5} m.) the values become 0·12 and 1·94 respectively. That is, the depressant effect increases more rapidly than expected.

There are at least two factors which may make the depressance, owing to substances such as HCN and H_2S, more complicated. In the first place the relative depressant effect of the ions and molecules of these weak acids is not known. It seems probable that, unless the inhibitor is being destroyed at the assimilatory centres, a possibility not to be neglected, the concentration of the molecules will be the same at the assimilatory centres as outside the cell. The concentration of the ions, however, will decrease near the chloroplast as the rate of assimilation decreases, since the chloroplast which is consuming carbonic acid will tend to be more alkaline than the outside of the cell, and more so the greater the rate of assimilation. If the ions have a depressant effect then the total depressant effect will not increase as rapidly with increase in the outside concentration of inhibitor as it would if only the molecules were effective. Secondly, there is the possibility that the respiration in the illuminated cell is much greater than in the darkened cell (*cf.* Appendix), and more so the greater the rate of assimilation. If this is so, and this respiration, like that of the darkened cell, is increased by dilute concentrations of HCN and H_2S, then clearly the relation between net oxygen production plus respiration in the dark and concentration of inhibitor may be complicated.

Whatever may be the explanation of the departure of the results from expectations based on the simple formulation of the schema there is one

* The temperature for the last three experiments was 25° C., for the others 20° C.

respect in which we may have made our formulation too simple. We assumed that the rate of breakdown of S_l to give the products of assimilation was proportional to $B \,.\, S_l$. If this reaction is like the majority of enzyme reactions which have been investigated then B and S_l will combine to form a complex X, which will break down to give the products—only in the limit when the velocity constants of the reactions involving the breakdown of this complex are very great compared with the velocity constant of the reaction involving its formation will the rate of assimilation be proportional to the product of the concentrations of B and S_l. In other cases the amount of free S will be $(S - S_l - X)$ and not $(S - S_l)$ and the effective amount of B will be reduced to $B - X$. We then have

$$dS_l/dt = k_1 \,.\, L(S - S_l - X) - k_2 \,.\, S_l\,(B - X) - k_3 \,.\, S_l + k_4 \,.\, X.$$

The first term represents the activation of S, the second the combination of activated S with free B, the third the reversion to the unactivated form, and the fourth the formation of S_l by the dissociation of the compound with B.

$$dX/dt = k_2 \,.\, S_l\,(B - X) - k_4 \,.\, X - k_5 \,.\, X.$$

The first term gives the rate of combination of S_l with B, the second that of the reversion and the third the rate of assimilation. When the steady state is reached

$$X = k_2 \,.\, S_l \,.\, B/(k_2 \,.\, S_l + k_4 + k_5)$$

and

$$S_l = \frac{k_1 \,.\, L(S - X) + k_4 \,.\, X}{k_1 \,.\, L + k_3 + k_2 .\, (B - X)}.$$

As far as the effect of change of B by depressants is concerned the relative depression at high and low intensities of illumination is the same as in the simpler formulation. As L is reduced X approaches the value

$$X = k_1 \,.\, k_2 \,.\, L \,.\, S \,.\, B/(k_4 + k_5)(k_3 + k_2 \,.\, B).$$

Provided k_3 is small compared with $k_2 \,.\, B$, that is the spontaneous reversion of S_l is negligible compared with its combination with B (the high energetic efficiency of the assimilation process at low intensities of illumination suggest that this must be so), then a decrease of B has no appreciable effect on the rate of assimilation. As L is increased X approaches the value

$$X = \frac{k_2 \,.\, B(S - X)}{(S - X) + k_4 + k_5}.$$

In this state X does decrease with B, but not proportionally, since $S - X$

increases. The decrease of rate only becomes proportional to the decrease of B when this quantity is made so small that X, to which the rate of assimilation is proportional, is made so small that it is a very small fraction of S. As we have pointed out, the situation is such that we cannot say whether this is the type of relation between the rate of production of oxygen in the assimilatory process at high intensities of illumination and the amount of effective B substance, as it is gradually reduced by the addition of inhibitors such as HCN. As we have seen, there are suggestions that over certain ranges of concentration of inhibitor the depressant effect increases more rapidly than our simple formulation would lead us to expect. This is the relation our more complete formulation suggests.*

Adopting the above formulation as a working basis for the mechanism of photosynthesis and incorporating it in our schema to account for the induction phase observed in our experiments, we must bear in mind the fact that the rate of assimilation must change with time in such a manner that $\log(1 - R/R_\infty)$ when plotted against time gives a curve the slope of which increases during the early stages, when the concentration of inhibitor is relatively great—a fact for which our first schema did not allow.

We assume the production by the cell of an inhibitor, of the HCN type, at a rate k_6Y, and its removal during assimilation. The first question to be settled is the relation between the rate of assimilation and the rate of removal of the inhibitor. The first possibility we examined was that the rate of removal was proportional to the oxygen concentration in the cells, which under our experimental conditions means proportional to the rate of assimilation, since the oxygen concentration outside the cells was practically zero. This assumption as to the rate of removal did not fit the facts as to the relation between rate of assimilation and time. It does not seem likely on other grounds. During the dark period prior to the experiment the atmosphere around the cells contained 5 per cent. or so of oxygen. For various reasons it does not seem likely that the resistance of passage of oxygen to and from the cells of the thin leaf is high enough to cause the oxygen concentration to be much different from that outside the cells even when there is the relatively rapid traffic in oxygen that there is during assimilation. Hence we think that the oxygen concentration in the cells was higher in the dark than it was during illumination. This being so, a substance, the destruction of which was increased by increase

* The above picture of the mechanism of photosynthesis is in good agreement with the other known facts of photosynthesis, but this is not an occasion to discuss the agreement in detail.

in oxygen, would tend to accumulate in the light faster than in the dark. We turned then to the hypothesis that the inhibitor was destroyed at a rate proportional to the concentration of S_l; Willstätter and Stoll's hypothetical peroxide form of the compound of chlorophyll and CO_2.*

Second Schema.—This, then, is our schema. A complex of some substance we will call S (which may be chlorophyll) and carbon dioxide is converted to S_l as the result of absorption of light energy.† This latter substance, perhaps a peroxide form, may be broken down by a catalyst B to give carbohydrate and oxygen, or alternatively it may decompose an inhibitor which is produced by the cell and which combines with B, but not competing with S_l, and acts as a depressant. The only novelty is that the cell can itself produce the inhibitor. If the inhibitor is anything like as potent as H_2S only a very small concentration would be required to cause the depressions observed. We do not suggest that this is a picture faithful in every detail, but as we shall see it accounts for the present facts, and moreover suggests further lines of attack. To make the schema amenable to simple mathematical treatment we have made some assumptions which may not be completely justifiable.

We add to our previous expressions that the effective amount of B is

$$B_e = B \,.\, k_i \,/(i + k_i)$$

and that

$$di/dt = k_6 \,.\, Y - k_7 \,.\, S_l \,.\, i.$$

We assume that all the reactions other than the change of inhibitor attain steady states very quickly and that the changing rate of photosynthesis is an expression of the changing concentration of inhibitor.

With our expressions for X, to which the rate of assimilation is proportional, and S_l we could now derive expressions for the rate of assimilation and for the amount of assimilation as a function of time. The expression for X is,

$$X = k_2 \,.\, S_l \,.\, B_e/(k_2 \,.\, S_l + k_4 + k_5),$$

* Gaffron (1927) has shown that allyl thio-urea is oxidized by an acetone solution of ethyl chlorophyllide in the light but not in the dark, the photo-oxidation of the chlorophyll derivative being prevented. It is not assumed that our hypothetical inhibitor is not destroyed by oxygen but that it is destroyed more rapidly by S_l.

† We favour this type of picture rather than one similar to Warburg's first type, because the experiments of Emerson and Arnold (1932) with intermittent illumination, and some recent experiments by Mr. H. G. Wager, show that change of CO_2 concentration acts in the same way as change in intensity of illumination, thus suggesting that light and CO_2 act on the same part of the mechanism.

B_e is substituted for B in the earlier expression. But that for S_l becomes

$$S_l = \frac{k_1 . L(S - X) + k_4 . X}{k_1 . L + k_3 + k_2 . (B_e - X) + k_7 . i}$$

not only does B become B_e, but there is an added term in the denominator to allow for the breakdown of S_l by reaction with the inhibitor. As we have already pointed out we have postulated a type of inhibitor which causes a greater depression at high intensities of illumination than at low. If the ratio of the amount of inhibitor at the commencement of illumination to that when a steady rate of assimilation has been attained was independent of the intensity of illumination then the ratio of initial rate to final rate would be greater the weaker the illumination. There is another factor which will tend to make the ratio yet greater. The weaker the illumination the less rapid will be the destruction of the inhibitor and hence the greater the amount when a steady state is reached: the change in the amount of inhibitor and in the rate of assimilation will be smaller.

Since, however, the greater the intensity of illumination the more rapidly does the inhibitor fall to its steady value it is only in the very early stages that the ratio of rate to final rate is necessarily greater the weaker the illumination. As we have seen this holds as far as the average rate for the first 10 minutes is concerned.

Apart from the effect of temperature on the initial and final amounts of inhibitor, increase of temperature acts like decrease of illumination, since an intensity of illumination which is so high that further increase has little effect on the rate of assimilation at one temperature becomes relatively low, that is an increase of intensity has a definite effect on the rate, when the temperature is increased. This is an experimental fact and is in agreement with our schema. Hence the ratio of initial rate to final should rise as the temperature is increased. The final amount of inhibitor is $k_6 . Y/k_7 . S_{l\infty}$. If the velocity constant of formation, k_6, and that of destruction, k_7, are equally affected by change of temperature then the final amount of inhibitor ought to increase as the temperature is increased, for $S_{l\infty}$ will fall as the temperature is increased, except in the limit when the intensity of illumination is so great that S_l approaches its limiting value of $(S - X)$. This effect will tend to increase yet further the rise of the ratio of initial rate to final as the temperature is raised. As we have seen this increase of the ratio is what is observed experimentally between 11° and 25° C. As with the other schema the fall of the ratio beyond 25° can be attributed to the increase in the concentration of the inhibitor at zero time,

consequent upon an increase in the rate of production of the inhibitor during the preliminary dark period. As stated before these two factors, one tending to increase the ratio as the temperature rises and the other tending to reduce it, will be at work over the whole range of temperature. Over part of the range one effect may predominate and over another part the other effect: it is the ever-present phenomenon in biological reactions of the optimum temperature.

We have, then, in the present schema one which is as good as the previous one in accounting qualitatively for the relation between the ratio of the rate in the first 10 minutes to the final rate and temperature and intensity of illumination. We must now examine it to see if it gives a better account of the change of rate with time; the point where our first schema failed.

The ideal procedure would be to find the expression for the relation between the amount of assimilation and time, since this is the form our results take. Except for special cases this is difficult and even in these the process of comparison of calculated and observed results is very lengthy. Our attack will be less direct.

We have already noted that the curve of $\log(1 - R/R_\infty)$ or $\log P$, as we shall call it, against time shows an increasing slope during the early stages. Our first point will be to ascertain whether such a relation is to be expected on the basis of the present schema.

Putting

$$a = k_2 S_l/(k_2 . S_l + k_4 + k_5)$$

(p. 23) that is the final rate

$$R_\infty = k_5 . a . B_\infty$$

we have

$$P = 1 - a . B_e / a_\infty . B_\infty$$

a_∞ and B_∞ are the values to which a and B_e approach as time increases.

$$- d \log_e P/dt = \frac{a . B_e}{a_\infty . B_\infty - a . B_e} . \left(\frac{1}{B_e} . dB_e/dt + \frac{1}{a} . da/dt\right).$$

The relative rate of change of a; that is, $1/a . da/dt$, is less than that of S_l, and more so the greater S_l, the greater the intensity of illumination. The actual drift of S_l with time depends upon many factors since

$$S_l = \frac{k_1 . L(S - X) + k_4 . X}{k_1 . L + k_3 + k_2 . (B_e - X) + k_7 . i} \qquad \text{(p. 23).}$$

The quantity X, like the rate of assimilation, increases with time. Provided $k_4 . X$, the reversion of X to S_l and free B_e, is negligible the numerator of the

above expression will decrease with advance of time. Since X will not increase as rapidly as does B_e the denominator will tend to increase with time. The total result of these changes will be to make S_l decrease. The drift of S_l will depend also upon whether the changes of $k_7 . i$, owing to decrease of inhibitor, are negligible or not. The direct effect of decrease of inhibitor will be to make S_l rise with time.

In the limiting case where the intensity of illumination is very great S_l will be at the maximum value of $(S - X)$ throughout the whole time. If X is small compared with S then S_l will not fall as X increases with time. We shall then have a constant and

$$- d \log_e P/dt = \frac{dB_e}{dt} . \frac{1}{B_\infty - B_e} .$$

Even though S_l may change with time yet the change of a, which is much less if $k_2 . S_l$ is great compared with $(k_4 + k_5)$, may be negligible compared with that of B_e. Then putting B_e in terms of B and I and

$$di/dt = k_7 (S_l . i - S_{l\infty} . i_\infty)$$

we have, since S_l is assumed to change little

$$- d \log_e P/dt = k_7 . S_l (i_\infty + k_i)/(i + k_i).$$

The slope of log P therefore increases with time as the concentration of inhibitor falls. This the result obtained in our experiments. If S is not great enough to make the change in S_l inappreciable then the change in the slope of the log P curve will not be so great.*

What happens to the slope of the curve at lower intensities of illumination depends upon the direction and extent of the changes in S_l and a. Later we shall produce evidence suggesting that S_l falls with time, and as we have seen there are theoretical grounds for expecting this. Provided the change in S_l is still small enough to neglect the percentage change of a compared with that of B_e we have

$$- d \log_e P/dt = \frac{i_\infty + k_i}{i + k_i} . \frac{k_7 . (S_l . i - S_{l\infty} . i_\infty)}{i - i_\infty} .$$

If the percentage change of S_l is small enough compared with that of i the slope of the log P curve can still increase, since the decrease of the second component

* That some photosynthetic cells have S in great excess is suggested by the fact that leaves of yellow varieties have practically the same assimilatory activity when the illumination is high as the leaves of green varieties of the same species with a far greater concentration of chlorophyll (Willstätter and Stoll, 1918).

of the above expression can be less than the increase in the first component, $(i_\infty + k_i)/(i + k_i)$. It is clear then that our present theory, in contrast with our first can account for the changing slope of the curve of $\log(1 - R/R_\infty)$ against time.

There is another way by which we could distinguish between the relative merits of the two theories if we had sufficient data. According to the first schema the slope of the $\log_e P$, time curve should be constant at

$$-k_2 \cdot \frac{L + k_l(1 + i/k_i)}{L + k_l}.$$

That is it should decrease with increase in the intensity of illumination. According to the second schema the limiting slope to which the curve approaches

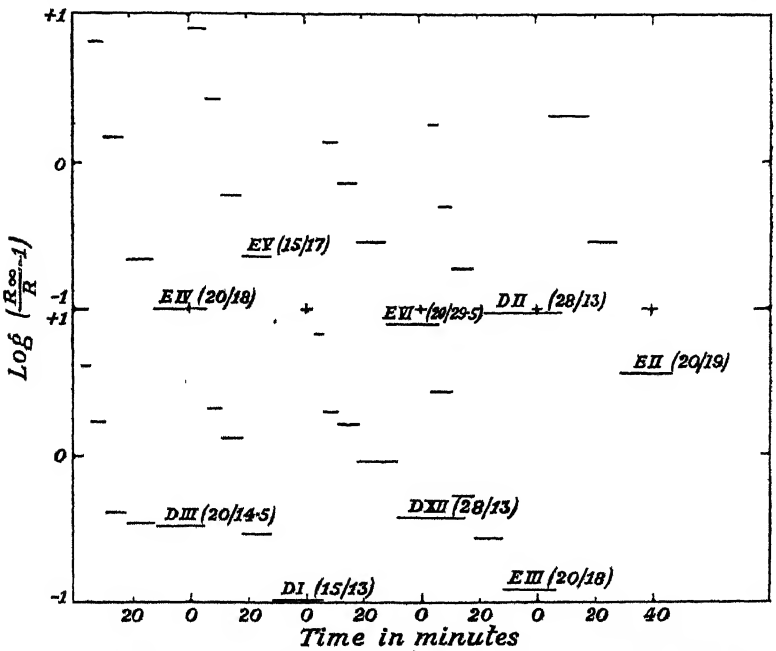

FIG. 5.—Log $(R_\infty/R - 1)$ and time for nine experiments. (See Note, Fig. 4.)

is proportional to $S_{l\infty}$, a quantity which increases with the illumination. To decide this point we should require many more data at one temperature because of the difficulty of deciding the values of P as they approach zero, and because the final slope depends upon the amount of inhibitor, a factor, at present, beyond our control.

The second quantity we are going to consider is $(R_\infty/R - 1)$ or M as we shall call it. As the curves in Fig. 5 show, the slope of log M against time decreases,

but it approaches more closely to a straight line the higher the intensity of illumination, provided the temperature does not go too high, *cf.* E.V and D.I.

Neglecting the change in a compared with that in B_s, as before, it can be shown that

$$-d\log_e M/dt = k_7 \,.\, (S_l \,.\, i - S_{l\infty} \,.\, i_\infty)/(i - i_\infty)$$

since

$$d\log M/dt = d\log P/dt \,.\, B_\infty/B.$$

The slope approaches the limit $k_7 \,.\, S_l$ as time increases and provided S_l decreases with time (see previous discussion) the slope will decrease with time. As the illumination is made greater the change of S_l becomes smaller, and, as we have shown, if S is great compared with X the change of S_l becomes negligible and the slope of the curve is constant. So it is seen that the data, as far as the qualitative changes of rate with time are concerned, can readily be made to fit in with the present schema.

For the limiting case at high illumination where the slope of $\log_e(R_\infty/R - 1)$ against time is practically constant at $k_7 \,.\, S_l$ we have a simple relation between the rate and the time, and the relation between the amount of assimilation and time can be readily obtained in a simple form. We can, then, make a direct and strict comparison between the experimental results and the theoretical expectations. So far our comparisons have been approximate, since the values of rate have been for intervals of time during which the rate has been changing in an unknown manner and hence the curve of log M against time could not be plotted accurately.

Experiments E.V and D.I with high illumination and temperatures of 17° C. and 13° C. respectively show the closest approach to linearity of the log M, time relation. For the same illumination at higher temperatures the illumination is more remote from that region where further increase of illumination has little effect on the rate of assimilation, and hence the observed departure of the curves from linearity is not surprising. We propose to compare the observed values of assimilation for E.V and D.I with the values calculated from

$$d\log_e M/dt = -k_7 \,.\, S_l$$

on integration between the limits t and 0, we get for the assimilation, A,

$$A = R_\infty \,.\, t - \frac{R_\infty}{k_7 \,.\, S_l} \,.\, \left[\log_e \frac{R_\infty}{R_0} - \log_e(1 + M_0 e^{-k_7 \,.\, S_l \,.\, t})\right]$$

M_0 is the value of M at $t = 0$.

Table III.—Observed and Calculated Values of Assimilation.

	D.I.		E.V.	
	Real assimilation.	Apparent assimilation.	Real assimilation.	Apparent assimilation.
Final rate, R_∞	3·1	2·85	2·95	2·87
R_∞/R_0	7·08	18·6	26·1	21·8
$k_7 . S_l$	0·116	0·17	0·24	0·208

D.I.					E.V.				
Time.	Real assimilation.		Apparent assimilation.		Time.	Real assimilation.		Apparent assimilation.	
	Observed.	Calculated.	Observed.	Calculated.		Observed.	Calculated.	Observed.	Calculated.
min.					min.				
6	2	3	1	2	6	2	1	1	2
11	7	7	5	5	11	6	5	5	5
18	17	17	13	14	18	19	17	17	15
28	41	39	35	34	28	43	43	39	39
46	91	88	82	83	47	100	99	93	93
58	126	124	114	117	67	159	158	150	150
126	334	334	310	310	91	229	229	217	219

Assimilation unit 0·01 c.c.

The value of the final rate is obtained from the data and, by trial, values of $k_7 . S_l$ and R_0 were obtained which would give a satisfactory agreement of the calculated and observed values of assimilation. The results are recorded in Table III. It is not claimed that the values of R_0 and $k_7 . S_l$ chosen are those giving the best agreement. In E.V the difference is never greater than 0·02 c.c. —errors as big as this may occur through lack of precision in the barometer and thermometer readings. In D.I there is only one difference of more than 0·02, namely, 0·03. To the observed values for oxygen production values for respiratory consumption from observations on the consumption in the dark were added. Provided these figures for respiration are not greatly different from the figures for the respiration of the illuminated cells as good an agreement of calculated and observed values for assimilation can be obtained. In Table III are the values when no addition has been made for respiration. The agreement is of the same order as before and although the values of $k_7 . S_l$

and R_0 are necessarily different from what they were before, yet $k_7 \, . \, S_l$ still is greater for E.V than for D.I.

There are a few points of interest with regard to the difference between the constants for the two sets of data. If the material had been identical in all respects one would have expected the final rate to have been higher for E.V at the higher temperature; actually the apparent assimilation was about the same, but the real assimilation a little lower. As we have seen even the same material may give different final rates under the same conditions. The differences for the same material may be due to differences in S, B, or the amount of inhibitor which is present when the rate of its destruction equals its rate of formation. With different lots of material the amount of material is an extra factor.

There are marked differences between the experiments in respect of the values of R_∞/R_0 and $k_7 \, . \, S_l$. These values are fairly close, if not identical with those giving the best agreement of the calculated and observed values for assimilation. If the change of S_l during the experiments is negligible, then the ratio of the rates of assimilation will be a measure of the ratios of the amount of B substance free from inhibitor, that is R_∞/R_0 is equal to $(i_0 + k_i)/(i_\infty + k_i)$. The greater value of this ratio for E.V, at 17° C., compared with that for D.I, at 13° C., may be due partly to a greater value for i_0, the concentration of the inhibitor at the outset,* partly to a lower value of k_i, if the combination of B and inhibitor is endothermic, and partly to a lower value of the final amount of inhibitor, i_∞.

It is quite clear from the above that our second theory gives a good quantitative account of the relation between assimilation and time when the illumination is relatively high. That our first theory would fail again particularly in the early stages, is shown by the increasing slope of the log $(1 - R/R_\infty)$ curve (see fig. 4), which according to the first schema should be constant.

Our present theory in its general form is too complex to make any quantitative comparison for lower intensities of illumination. For the special case where X is small compared with S and B_s, which seems to us to be what Warburg means in his first schema, the quantity a of our general formulation becomes S_l. Then, for high illumination the slope of the $\log_e$ M, time curve becomes $k_7 \, . \, S$ instead of $k_7 \, . \, S_l$, and we have the same agreement of calculated and observed values of assimilation as before, but our constant $k_7 \, . \, S$ has a different

* We suggested earlier that quite possibly the initial amount of inhibitor might increase with increase of temperature.

significance. For other intensities the slope of the $\log_e M$, time curve can be readily deduced.

$$-d \log_e M/dt = k_7 . (S_l i - S_{l\infty} . i_\infty)/(i - i_\infty)$$

as in the general formulation, but here

$$S_l = k_1 L . S/(k_1 . L + k_3 + k_2 . B_e + k_7 . i).$$

This approximates to $k_7 . S_l$ since the percentage changes of S_l are smaller than those of B_e, which are smaller than those of i.

Putting f for the ratio of the final rate to that when the illumination is great, in both cases there being no inhibitor,* and g for the ratio of the actual final rate to that which would be attained if there was no inhibitor left when the steady rate is reached, we obtain the following relation between S_l and rate

$$S_l/S = 1 - g . R(1 - f)/R_\infty$$

and hence

$$\log_e M_0 - \log_e M = k_7 . S\{t - g . A(1 - f)/R_\infty\}$$

where A is the total amount of assimilation at any time, t. That is log M should give a straight line when plotted, not against time, but a corrected time obtained by subtracting a quantity proportional to A/R_∞ from the actual time.† This linear relation can be realized for the experiments with lower intensities of illumination, but clearly, since we have two variables f and g to conjure with and no other certain criterion as to whether we have chosen appropriate values, except that we have obtained the linear relation, this analysis cannot help us very much. So we must rely chiefly upon the agreement of fact and theory for the conditions when the illumination is great as an indication of the numerical sufficiency of our schema.

Examination of the Results of other Workers.

Finally, we will examine the data provided by some other workers to see if they agree with our schema. Paauw (1932) states that the assimilation of *Hormidium* showed a negligible change of rate after the first minute or so, but proceeds "however, when I look through my protocols it is evident that there exists an initial increase, after a long stay in the dark and also in the dark winter months." Since these changes in rate were not the rule it does

* The final rates are $k_1 . k_2 . L . S . B . /(k_1 . L + k_3 + k_2 . B)$ and $k_2 . S . B$.

† As with the general formulation the slope of the log M curve decreases : $A/R_\infty . t$ rises as t increases.

not seem likely that the phenomenon has anything to do with the technique of measurement of assimilation. Paauw gives the results of several experiments showing an induction phase, but only one provides data giving more than two values of $(R_\infty/R - 1)$ greater than zero. In Table IV are given the observed values of the assimilation for successive intervals and those calculated on the assumption that $\log (R_\infty/R - 1)$ is a linear function of time. This is the relation we should expect according to our theory since the illumination was maximal. The agreement with the theory is clearly very good. It would, however, have been more satisfactory if there had been data nearer zero time than 15 minutes, for as we have seen it is in the early stages, when the rate is changing rapidly, that a theory may breakdown. The value of $k_7 . S_l$ (0·076) is lower than that for our two experiments done at a lower temperature—the temperature in Paauw's experiment was 20° C. This is not remarkable for the expected increase with temperature may be more than balanced by a smaller value for S_l or an inhibitor that is destroyed less easily, that is k_7 smaller.

Table IV.—Assimilation by *Hormidium* (Paauw), Observed and Calculated.

Time interval.	Calculated $R_\infty/R_0 = 3·24$ $k_7 . S_l = 0·076$.	Observed.
minutes		
15–20	22	22
20–30	52	54
30–35	29	29
35–40	31	31
40–45	31	32

There is the set of data provided by Osterhout and Haas to which we made reference in the introduction. These should not be accepted as evidence of a real induction phase in the photosynthetic process itself without further examination. It is not clear from the experimental detail that efficient precautions were taken to eliminate an induction phase in the rate of absorption of carbon dioxide owing to the establishment of diffusion gradients from the surrounding sea water into the tissue. Further, the absorption of carbon dioxide was measured by the change in the hydrion concentration of the sea water, and, as we shall show elsewhere, there are other factors at work during the early stages which tend to alter the hydrion concentration of the surrounding medium. Nevertheless, it is instructive to test the agreement of the results with our

theory if only to make us more cautious in drawing conclusions as to the validity of a theory based upon the agreement of an isolated set of data with figures based upon the theory. The actual data are for apparent assimilation, but Osterhout and Haas compared them with values calculated from an equation for real assimilation. There are no data for respiration, but, as we have seen for our results, provided the respiration is reasonably small compared with the apparent assimilation, using apparent instead of real assimilation makes no essential difference to the agreement of the calculated and observed values; only the values of the constants are altered. In Table V are the observed values, those calculated by Osterhout and Haas, and those calculated

Table V.—Assimilation by *Ulva* (Osterhout and Haas), Observed and Calculated.

Time.	Observed.	Calculated from our expression $R_\infty/R_0 = 2\cdot35$ $k_7 . S_l = 0\cdot0318$.	Calculated from expression of Osterhout and Haas.
minutes			
35·7	1	0·99	0·92
61·6	2	1·98	2·07
84·9	3	2·98	3·18
106·6	4	3·98	4·23
127·0	5	4·96	5·23

on the basis of our theory taking the illumination, which was full sunlight, as great. The agreement with our calculated values is good, far better than with the other calculated values. It is interesting to note that when expressed mathematically the theory of Osterhout and Haas is identical with our first schema and postulates a linear relation for $\log(1 - R/R_\infty)$ against time, whereas the agreement with our calculated values shows that the $\log(R_\infty/R - 1)$ is more nearly linear. After our remarks about the induction phase possibly arising from causes outside the assimilatory process, too much stress should not be laid upon the difference between the value of $k_7 . S_l$ for this experiment and that for ours. If this quantity, 0·0317, really is an indication of $k_7 . S_l$ at the relatively high temperature of 27° C. it may be an indication that the velocity constant of the destruction of inhibitor, k_7, is here small. On the other hand, according to the view that S_l is an activated complex of chlorophyll and carbon dioxide as we have suggested earlier, we should expect S_l to be much smaller in this case than in our experiments, since here the

concentration of carbon dioxide was very small, namely, that in ordinary sea water.*

We now turn to the experiments of Warburg, where the induction period was over in the space of a minute or so. Again the illumination was great so that we can compare the results with those expected if $\log (R_\infty/R - 1)$ is a linear function of time. The results of the comparison given in Table VI show that the agreement could hardly be better. The very high value for $k_7 . S_l$, which is the cause of the short induction phase, may mean a high value for S_l, or, perhaps rather, an easily destroyed inhibitor.

Table VI.—Assimilation by *Chlorella* (Warburg), Calculated and Observed.

Time.	Calculated $R_\infty/R_0 = 7 \cdot 17$ $k_7 . S_l = 3 \cdot 06$.	Observed.
minutes.		
0·5	2·1	2
1·0	7·1	7
1·5	14·1	14
2·0	21·8	22
3·0	37·8	38

Paauw did experiments which in some respects were similar to those of Warburg, but actually they are more complicated. Warburg made observations on the amount of assimilation in 0·5, 1·0, 1·5, 2·0 minutes and so on, in each experiment there was a preliminary dark period of 5 minutes. In Paauw's experiments the illumination was intermittent, that is the assimilation in several periods of illumination was measured together, and further the dark period varied with the length of the light period, from 4·9 to 9·5 minutes. Warburg has shown that in assimilation with intermittent illumination, the light period being 1 minute, the assimilation decreased with increase of the length of the dark period. The amount was less with 4 minutes than it was with 3, but no data are given for any longer period of darkness. We have no knowledge as to whether with intermittent light such as that used by Paauw

* The fact that these data for an induction phase, although probably complicated by factors other than those allowed for in our schema, yet agree in form with the schema, has little weight as evidence against the schema, which applies to a wide range of data not subject to these complications. It merely shows how misleading a single agreement of fact and theory may be. If there is, here, an induction phase of the type suggested for our experiments then any further lowering of the rate in the early stages must operate in such a manner as to leave $\log (R_\infty/R - 1)$ still a linear function of time. The slope (apparent $k_7 . S_1$) will obviously be decreased by the complicating factors.

the amount of assimilation in successive periods of illumination is the same right from the commencement. If it is not, the average rate would vary with the number, which in these experiments is only a few. As the data are such that it is not easy to make calculations the results are presented in fig. 6 in the form of curves for log $(R_\infty/R - 1)$. The values beyond 1·5 minutes are not reliable because the rate approaches so closely to the final rate. There are only two points for the experiment at 26° C., since after 1·5 minutes the average rate fluctuates violently, rising to 18 and falling to 5·6, and rising again to 22·1. Even the average rate from 1·5 to 3 gives 16·95, while the rate with continuous illumination was only 16·2. It will be noticed that the relation

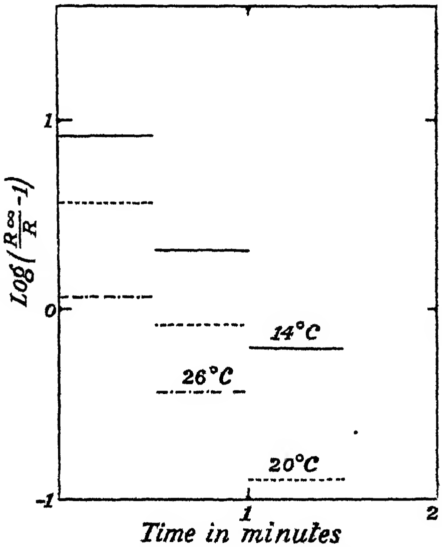

Fig. 6.—From data for *Hormidium* (Paauw).

approximates to linearity and that the slope at 14° C. is less steep than that to 20° C., as our theory suggests. Further, as in our experiments up to 25°, R_0/R_∞ rises with temperature, that is $(R_\infty/R_0 - 1)$ falls.

This survey of other data lends support to our explanation of the induction phase. If our schema is wrong then it would appear that the correct schema has a similar mathematical dress.

Finally we must refer to the work of Willstätter and Stoll on the assimilation of leaves of various plants in atmospheres containing no oxygen. The material was under anærobic conditions in the dark prior to the experiment, although the experiments are supposed to be on the effect of absence of oxygen during assimilation. They found that leaves such as those of *Pelargonium* are killed under these conditions. We found this effect even when the oxygen was not

removed until after the illumination was begun. This suggests that there can be very little oxygen in the leaf cells during illumination. The plants that withstood the conditions showed a lower assimilation in the first 20 minutes of illumination than they did subsequently. The initial depression was greater the longer the period of exposure to anærobic conditions. The author claim that their results show that oxygen is essential for assimilation, that a small quantity suffices and " dass nicht freier Sauerstoff, aber dissoziabel gebundener für die Assimilationreaktion unentbehrlich ist." The increasing depressions with length of preliminary period of exposure to anærobic conditions is the evidence adduced in support of the latter conclusion. The initial rate falls from 37 to 12 to 4 as the exposure is lengthened from 3 to 17 to 40 hours. As to whether this continued fall is duo to irreversible depression there are no data to decide, but as the figures stand it looks as if it took many hours of anærobic respiration to get rid of the dissociable compound of oxygen.

They make no precise formulation of the way in which the necessary oxygen acts, and there are not sufficient data to test any schema. Any simple formulation of an autocatalytic schema such as they suggest, where the assimilation rate increased with the concentration of oxygen produced in the process, would necessitate a more marked induction period the weaker the illumination, and could not explain the results of Warburg, or ours. Unfortunately Willstätter and Stoll limited themselves to one set of conditions. If the inhibitor causing the induction period was of the same type in their experiments as in ours, then it appears that anærobic conditions favour its production. In this connection it should be noticed that in our experiments although the plant material was not deprived of oxygen in the dark prior to the experiment, yet the concentration was usually less than that in the air ; usually it was about 4 or 5 per cent. When it was exposed to anæroboic conditions there was a prolonged induction period. Further the experiments of Paauw and that of Osterhout and Haas, in which fairly long induction phases are recorded, are after long periods of darkness and they were obtained with plants in water—conditions which might tend to shortage of oxygen in the cells. On the whole it looks as if the production of the inhibitor might have some connection with anærobiosis. This is a question which requires further investigation and may lead to a method of controlling the amount of inhibitor, thus making smaller the number of results necessary for a satisfactory analysis.

For brevity we have left out most of the detailed steps of the mathematical deductions ; the assumptions are stated and the final expressions used can be obtained in a simple manner.

Summary.

This paper may best be summarized, not by an outline of the results and the schemata put forward to account for them, but rather by a concise account of the method of attack. In the first place the relation between the ratio of the rate of assimilation in the early stages of the induction phase to the final rate and the intensity of illumination and temperature was used in a preliminary survey to ascertain the type of schema required. This having been decided, we formulated the schema more precisely and tested whether it would account not only for the relation of the ratio to illumination and temperature, but also for the quantitative changes the ratio undergoes as the induction phase advances. The schema must also be one which is acceptable in the light of our knowledge of photosynthesis in particular, and of photochemical reactions generally. In this way we were able to eliminate certain formulations as being less probable. Finally, we arrived at a schema which gives a good quantitative account of the facts (our own and those recorded by other workers) and is in agreement with other known facts of photosynthesis. From this we may conclude, either that our schema approximates to a true picture of the mechanism involved, or that the correct explanation has a mathematical formulation which, for the range of conditions investigated, approximates closely to that of our present schema. The schema will be confirmed or modified as new facts accumulate; in the meantime it suggests new lines of attack.

The data for photosynthesis are so numerous that the time seems ripe for the more precise formulation of theories, many of which have been but vaguely suggested and rarely put to a quantitative test. The process of formulation, testing, and rejection or refinement, must necessarily be tedious for such a complicated biological reaction.

Appendix.

The Interaction of Respiration and Assimilation in Chlorella.

As already indicated experiments such as those reported in the present paper make the question of the magnitude of the respiration in a plant cell during assimilation one of particular interest.

Warburg records experiments showing that an increase of the partial pressure of oxygen from 1/50 atmosphere to 1 atmosphere decreased the assimilation of *Chlorella*. With a high intensity of illumination the depression varied from 41 per cent. to 73 per cent., while with a low intensity the effect was negligible. Warburg suggests two possible explanations. In the high intensity the assimilates are in a high concentration and are oxidized back to CO_2 before they can be

converted into a stable form. The other is that the photochemical primary product, that is, the light-activated substance, is destroyed by oxygen before it can react with the carbon dioxide. He makes no attempt to formulate a precise schema, he merely dismisses the first possibility as "der uninteressanter Fall," and draws attention to the many cases where oxygen depresses photochemical reactions. If he had referred to his mathematical treatment of his schema for photosynthesis he would have found that it is at low intensities of illumination that the rate of assimilation is proportional to the concentration of photochemical primary product, while at high intensities it is independent. Consequently the favoured second explanation implies a depression owing to increased oxygen which is greater the lower the intensity of illumination.

The former explanation can be made to fit the facts. Let us suppose that the first products of assimilation pass through a form, U, which is much more unstable in the presence of oxygen than the normal form of carbohydrate to which the unstable form changes. The two possible fates of this unstable form are oxidation or stabilization. It seems likely that the rates of these processes may be expressed by $k_1 \,.\, \mathrm{U}/(\mathrm{U} + k_0)$ and $k_2 \,.\, \mathrm{U}/(\mathrm{U} + k_s)$, where k_1 is a function of the oxygen pressure. When a steady state is attained the rate of photosynthetic fixation of carbon dioxide, A, will be given by

$$\mathrm{A} = k_1 \,.\, \mathrm{U}/(\mathrm{U} + k_0) + k_2 \,.\, \mathrm{U}/(\mathrm{U} + k_s)$$

and the apparent rate, B, by

$$\mathrm{B} = k_2 \,.\, \mathrm{U}/(\mathrm{U} + k_s).$$

To show the possible effect of the change of partial pressure of oxygen on the value of B, which is the only rate of assimilation which can be measured, we must assume relative magnitudes for the various constants. If k_2 is four times k_1 and k_0 twenty times k_s, then an increase of oxygen concentration which increases k_1 twenty-fold will depress the assimilation by 32 per cent. when the illumination is so high that U, at the low concentration of oxygen, is equal to 10 k_s, while it will decrease it by only 3·5 per cent. when the illumination is so low that U is small compared with k_0 and k_s.

Although we do not know the effect of change of oxygen pressure on k_1 it is clear that Warburg's results can be explained on the basis of the oxidation of the unstable early products of photosynthesis, and that this oxidation must be a relatively large fraction of the total production when the intensity of illumination is high. If this view approaches the truth, then allowance for respiration on the basis of oxygen consumption in the dark, when the unstable

substance is at a low level, gives a completely erroneous idea of the real rate of carbohydrate production in photosynthesis. The proper allowance for oxidation should vary both with the rate of production of carbohydrate and with the concentration of oxygen—any relation such as that between assimilation and illumination or assimilation and concentration of carbon dioxide will be dependent upon the concentration of oxygen. The latter is a direct conclusion from the experimental results apart from any theory of the mechanism. The complications of the question increase when it is realized that oxygen pressure outside tissue may be maintained constant, but the pressure at the seat of the oxidation processes, which is the determining factor, depends upon the rate of oxidation and upon the rate of production of oxygen in the photosynthetic process.

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The Permeability of Human Skin to Electrolytes.

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The epidermis is not only a protective covering against mechanical injury, but presumably protects the body also from diffusion inwards of foreign substances or diffusion outwards of normal constituents of the blood. In a previous paper (Whitehouse, Hancock and Haldane, 1932) it was shown that though the epidermis is permeable to water, and the actual permeation is under physiological control and plays an important part in the regulation of body-temperature, yet salts and colloidal substances do not appear to diffuse through the epidermis, so that in this respect the epidermis seems to play the part of a semi-permeable membrane. The question of the permeability of the epidermis to various substances which are met with in mining and other operations, or are applied with a remedial object, is of much practical interest, and in connection with the treatment of mining accidents from burning, the apparent impermeability of the outer epidermis to colloidal solutions of tannic acid and ionized solutions is of special importance. It is presumably the dense *stratum lucidum* of the external epidermis that tends to stop diffusion.

The present investigation deals with the question whether the skin is entirely impermeable to electrolytes in solution. The choice of suitable substances for the tests was necessarily limited to elements which are either not present in urine or blood, or present in only very small amounts, and which could also be detected and determined very accurately.

Lithium as a kation was chosen for the first series of experiments, since it is only present in very minute amounts in human blood and urine, and a very small increase in its concentration can be detected. A spectrographic method similar to that used previously by one of us (Ramage, 1929) was used to test for the presence of lithium. A measured volume of urine was run on to a piece of Munktell's ashless filter paper weighing about 0·11 gm. After drying in a steam oven, the paper was made into a tight roll and burnt in an oxy-coal gas flame issuing from a fused silica burner, the light being focussed by a quartz lens on the slit of the spectrograph. The piece of filter paper absorbed 0·1 c.c. of fluid easily and, if a larger quantity was necessary for the analysis, it was

added 0·1 c.c. at a time and the paper dried after each addition. Quantities up to 0·4 c.c. of urine and 0·2 c.c. of blood were used in the preliminary experiments and it was found that the best quantity to use was 0·1 c.c. and that as little as 0·00001 per cent. of lithium could be estimated. The spectrograms of the urine were compared with those obtained from measured volumes of a standard solution of lithium as described by Fox and Ramage (1931).

The first series of experiments was carried out by immersing the arm for a period of 1 hour in solutions of lithium chloride of varying concentrations. In order to increase the circulation of blood in the skin and thus facilitate any diffusion, the experiments were carried out in a chamber heated to about 90° F. The subject was seated in the chamber with one arm immersed in the lithium chloride solution, which was at about the temperature of the air in the chamber. The details are shown in the table.

Table I.

Experiment No.	Air temperature in chamber.		Temperature of lithium chloride solution. °F.	Concentration of lithium chloride solution Percentage by weight.
	Dry-bulb. °F.	Wet-bulb. °F.		
I	92	66	95·5	0·5
II	75	—	72·5	7
III	93	63	92·5	14

Samples of urine, and also of blood in experiments I and III, were taken before the start of each experiment and at intervals up to 24 hours afterwards. The results of the spectrographic analysis were as follows :—

Experiment I.—The quantity of lithium in the urine before the experiment did not exceed 0·00001 per cent., an amount it was possible to detect when 0·1 c.c. was taken for the analysis. The samples of urine collected after the arm had been immersed in the solution showed no increase.

The blood gave no evidence of lithium either before or after the immersion of the arm.

Experiment II.—In no sample did the quantity of lithium in the urine after the experiment exceed that originally present.

Experiment III.—Both the urine and the blood contained traces of lithium before the experiment, but in no case was there any increase afterwards.

In order to show how the spectrographic method of analysis would reveal a positive result as regards increase in lithium concentration of the urine, some further experiments were made in which very small quantities of lithium salts were taken through the mouth. One milligram of lithium (as lithium sulphate) was taken by mouth and the urine was collected at intervals. 0·1 c.c. of each urine sample was analysed spectrographically, and it was found that there was a distinct increase in the intensity of the red lithium line for about 7 hours following the dose. A better result was obtained when 5 mg. of lithium were taken, and a final experiment was made in which a dose of 43·5 mg. of $LiCl . H_2O$ (= 5 mg. lithium) was taken in about 150 c.c. of water. The results of the last experiment are given in Table II.

Table II.

Urine collected.	Specific gravity.	Volume c.c.	mg. lithium per 10 c.c. urine.
10 hours before dose	1·016	—	Merest trace
5 minutes before dose	1·024	—	Merest trace
0–1¼ hours after dose	1·025	34	0·08
1¼–2 ,,	1·025	22	0·10
2–5 ,,	1·021	136	0·06
5–7½ ,,	1·022	136	0·04
7½–9½ ,,	1·013	228	0·015
9½–14 ,,	1·024	400	0·02
14–23½ ,,	1·022	400	0·025
23½–26½ ,,	1·027	44	0·03

The weight of lithium recovered in the urine was approximately 4·1 mg., that is 82 per cent. of the lithium was eliminated in this way in 26½ hours.

The "traces" of lithium in normal urine and blood vary in amount from day to day, no doubt varying with the intake in the food. Some lithium is excreted in the fæces and some in perspiration. Although the quantities were too small to estimate, there was distinct evidence of lithium passing in the perspiration after a dose of 5 mg. of lithium (as sulphate), and in a less marked way of its presence in diminishing traces in saliva samples collected after the dose.

Since none of the blood or urine samples collected after immersion of the arm in lithium chloride solution showed any increase in lithium concentration compared with that of normal blood and urine, we conclude that the skin is entirely impermeable to the lithium kation.

Winternitz (1883), however, claimed to have detected traces of lithium in the urine after application of a solution of lithium chloride to the skin, the latter having been cleaned previously with ether. The details of his observations are not accessible to us, but in view of our results it seems probable that the lithium which he discovered had not been absorbed through the skin.

Following the negative result with lithium, the next step was to test the permeability of human skin to an anion. The most suitable anion appeared to be iodine and this was employed in the form of a solution of potassium iodide. Iodine is only present in minute amounts in normal urine, and very small quantities can be determined with reasonable accuracy. The method of determining the iodine in the urine samples was similar to that used by Leitch and Henderson (1926). The method consists briefly of drying and carefully ashing a known quantity of urine with potassium hydroxide, extraction of the iodide with 95 per cent. alcohol, oxidation with bromine-water, removal of excess bromine, addition of potassium iodide, and titrating with N/500 sodium thiosulphate solution after addition of starch.

In the first experiment the arm was immersed for an hour in a 5 per cent. (by weight) solution of potassium iodide at 95° F., the temperature of the air in the chamber being 84° F. dry-bulb, 68° F. wet-bulb. Examination of the urine showed no increase in iodine concentration, the results are shown in Table III.

Table III.

Sample of urine collected.	Specific gravity.	mg. iodine per 10 c.c. urine.
Before start	1·025	0·0008
0–1 hour after start	1·024	$<$0·0005
1–4 hours after start	1·025	$<$0·0005
4–9½ ,,	1·028	0·0006
9½–12 ,,	1·029	$<$0·0005
12–21 ,,	1·018	$<$0·0005

A second experiment, using a 1 per cent. solution of potassium iodide, gave a similar negative result.

Check experiments were made in a similar way to those with lithium, very small quantities of potassium iodide being taken through the mouth. The results are shown in Table IV.

Some recovery experiments in which a measured amount of potassium iodide was added to urine indicated, however, that the method was only roughly

quantitative, some iodine being evidently lost during the process of ashing. The method was therefore not suitable for determining the total iodine eliminated in the above experiments, but it was sufficiently sensitive, as shown in the table, to detect a distinct increase of iodine concentration in the urine after a dose of 1 mg. of potassium iodide (= 0·76 mg. iodine).

Table IV.

Experiment No.	Dose of potassium iodide taken through mouth.	Urine collected.	Specific gravity of urine.	Volume of urine c.c.	mg. iodine per 10 c.c. urine.
(1)	5 mg.	Before dose	1·023	—	0·0008
		0–½ hour after dose	1·018	46	0·0097
		½–1 ,,	1·005	130	0·0093
		1–3 hours after dose	1·015	150	0·0212
		3–5 ,,	1·016	130	0·0097
		5–9 ,,	1·011	370	0·0013
		9–12 ,,	1·018	250	0·0034
		12–21 ,,	1·010	700	0·0013
		21–24 ,,	1·022	110	0·0006
(2)	2 mg.	Before dose	1·023	—	0·0007
		0–½ hour after dose	1·016	32	0·0007
		½–1 ,,	1·010	62	0·0009
		1–2 hours after dose	1·007	180	0·0032
		2–3 ,,	1·015	96	0·0072
(3)	1 mg.	Before dose	1·024	—	0·0008
		0–½ hour after dose	1·025	22	0·0008
		½–1 ,,	1·026	16	0·0011
		1–2 hours after dose	1·025	60	0·0047
		2–3 ,,	1·015	74	0·0046

The elimination of iodine after the administration of various iodine compounds through the mouth has been studied by numerous investigators. With rabbits, Greenbaum and Raiziss (1927) found that potassium iodide, when administered in massive doses (about 1·4 gm.), was excreted in the urine within 5 to 6 days completely, or nearly so. Only traces of iodine were found in the fæces.

Although it is well known that oily substances are able to penetrate human skin, it was thought that some experimental data regarding permeability would be useful. A final experiment was made, therefore, in which approximately 1 gm. of iodine ointment (British Pharmacopœia 1914) was rubbed on the skin of the forearm, and urine samples collected during the following 21 hours. The results are recorded in Table V, and show clearly that un-ionized iodine is absorbed.

Table V.

Urine collected.	Specific gravity of urine.	Volume of urine, c.c.	mg. iodine per 10 c.c. urine.
½ hour before application	1·028	—	0·0015
0–2 hours after application	1·022	130	0·0017
2–5 ,,	1·026	142	0·0034
5–8 ,,	1·016	290	0·0038
8–12 ,,	1·021	400	0·0080
12–21 ,,	1·011	632	0·0017

Conclusion.

From the above experiments we conclude that the intact human skin is completely impermeable to electrolytes in simple solution.

Part of the expense of this investigation was covered by a grant from the Safety in Mines Research Board, to which acknowledgments are due.

In conclusion, the authors desire to thank Professor J. S. Haldane, F.R.S., for his valuable advice and interest in this work.

Summary.

It has been shown previously that the human skin is permeable to water, and that the actual permeation is under physiological control. The present investigation deals with the question whether the skin is entirely impermeable to electrolytes in solution. The method consisted of immersing the arm in solutions of varying concentration of (*a*) lithium chloride, and (*b*) potassium iodide, and examining the urine (and also the blood in some cases) before and at intervals up to 24 hours after immersion. In order to increase the circulation of blood in the skin and thus facilitate any diffusion, the experiments were carried out in a chamber heated to 75°–93° F., the solution being at approximately the same temperature. An extremely delicate spectrographic method was used for the determination of lithium, while iodine was estimated by a correspondingly delicate chemical method. In no case did the amount of lithium or iodine in the urine (or blood when examined) collected after immersion of the arm exceed the extremely small amount originally present. The conclusion is, therefore, that the intact human skin is completely impermeable to electrolytes in simple solution. An experiment in which iodine ointment was

rubbed on the skin showed a definite increase in the iodine content of the urine after application, and indicates that un-ionized iodine is absorbed.

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577 . 16 B_1 : 535 . 343 . 2

On the Ultra-Violet Absorption of Crystalline Preparations of Vitamin B_1.

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Evidence has been produced by several workers tending to support the view that vitamin B_1 possesses an ultra-violet absorption band with a maximum at about 260 mμ. Bowden and Snow (1932) stated that the preparation studied by them possessed such a band, which disappeared simultaneously with the vitamin activity on irradiation with monochromatic light of wave-length 256 mμ. The absorption curve published by Windaus *et al.* (1931) for their preparations also showed a maximum near 260 mμ. Heyroth and Loufbarrow (1932) have studied a large number of preparations of varying vitamin activity, and have shown that much of their ultra-violet absorption can be attributed to inactive purine and pyrimidine derivatives. Nevertheless, they conclude that there is a significant correlation between absorption at 260 mμ and biological activity (see also Ohdake, 1932).

It is therefore somewhat surprising that the preparations recently made at Oxford (Kinnersley, O'Brien and Peters (1932-1933)), which can claim to be the most potent yet produced, have their maximum absorption at a definitely different wave-length, namely 245–9 mμ, while at 260 mμ their absorption

curves only show a barely perceptible hump, protruding from the steeply descending portion of the main band. An attempt to connect this discrepancy with a possible effect of hydrion concentration on the wave-length of the maximum led to the discovery of a curious phenomenon. When an acid solution of the vitamin preparation was brought suddenly to about p_H 13 a broad band appeared at 330–340 mμ, together with an increase in absorption at the shorter wave-lengths, usually with a maximum at about 233 mμ. When the solution was heated for 2 minutes in the water bath the 330 mμ band disappeared. When the solution was made acid again the original absorption, with a single band at 245 mμ, was restored, practically undiminished in intensity. The same cycle was performed twice over on a single solution, with qualitatively the same result, suggesting that three substances, or three forms of a single substance, were undergoing reversible interconversion. Nevertheless, even after a single cycle the vitamin B_1 activity was substantially reduced. The simplest interpretation of these results is that the substance responsible for the 245 mμ band in the original preparation is not vitamin B_1. This interpretation, however, will only become necessary if it can be proved that the substances causing absorption in the preparation before and after an alkali cycle not only show similar absorption, but are identical. Perhaps the most interesting problem arising out of these results is that of finding a substance of known constitution which will behave towards alkali in a similar manner. The influence of hydrion concentration on the absorption of several purine derivatives and allied substances has been studied by Holiday (1930), with whom we have had the privilege of discussing this problem. Dr. Holiday has also made a special further study of ergothioneine, adenine, and hypoxanthine (private communication). It seems clear that none of these substances resemble the vitamin B_1 preparations in their behaviour towards alkali except in a few minor points.

The preparations examined by us absorbed about twice as intensely at 245 mμ as that of Windaus *et al.* at 260 mμ (1931). In consequence the hump which is just visible in the former at 260 mμ might mean that a considerable amount of the "260 mμ" substance was present together with that absorbing at 245 mμ. It is not at present possible to say whether the prominence of this hump varies significantly from one preparation to another.

Experimental.

The absorption curves were obtained with the apparatus described by Philpot and Schuster (1933). The changes in p_H were made by adding standard alkali

or acid from a micrometer syringe, or dropping tube, to the solutions in the quartz cell used for photographing their absorption spectra. The cell consisted of optical flats clamped together without cement. It therefore leaked slowly, making the exact calculation of concentrations impossible, and as no buffers were used the p_H values near the neutral point were very uncertain. Fortunately the chief change in absorption occurred well on the alkaline side, so that this crude technique, which was designed for rapid exploration with the use of a minimum of material, proved capable of giving a definite result.

Description of Curves.—The extinction coefficient is defined as :—

$$\varepsilon = \frac{1}{d} \log_{10} \frac{I_0}{I} - \varepsilon_0,$$

where d is the thickness of solution, I_0 and I are intensities of incident and transmitted light respectively, and ε_0 is the apparent extinction coefficient of the cell and solvent. The quantity ε is plotted as ordinates in all curves except in fig. 1, where ε/C is plotted, C being the concentration in grams per litre.

Fig. 1 shows ε/C for :—

(*a*) The preparation 61A (15) described by Kinnersley, O'Brien, and Peters (1933), dissolved in absolute alcohol containing N/200 HCl.

(*b*) The published curve of Windaus *et al.* (1931) for their preparation, replotted to the same scale as (*a*).

Fig. 2 shows ε for :—

(*a*) Preparation 61A (15) in 20 per cent. alcohol, which was used for the experiment below. It had previously been brought to about p_H 9–10 with very little change, and then made acid again. The 260 mμ hump is a little more prominent than in fig. 1 (*a*).

(*b*) The same solution made acid again after the treatment with alkali and heat described under fig. 3. The apparent loss of intensity agrees roughly with the dilutions undergone during the additions of acid and alkali. The dilution could not be calculated accurately for the reason given above.

Fig. 3 shows ε for :—

(*a*) The same solution as in fig. 2 (*a*) after addition of alkali to make approximately N/10 NaOH, showing the 330 mμ band well developed.

(*b*) The same after heating for 2 minutes in the water bath, with the 330 mμ band gone.

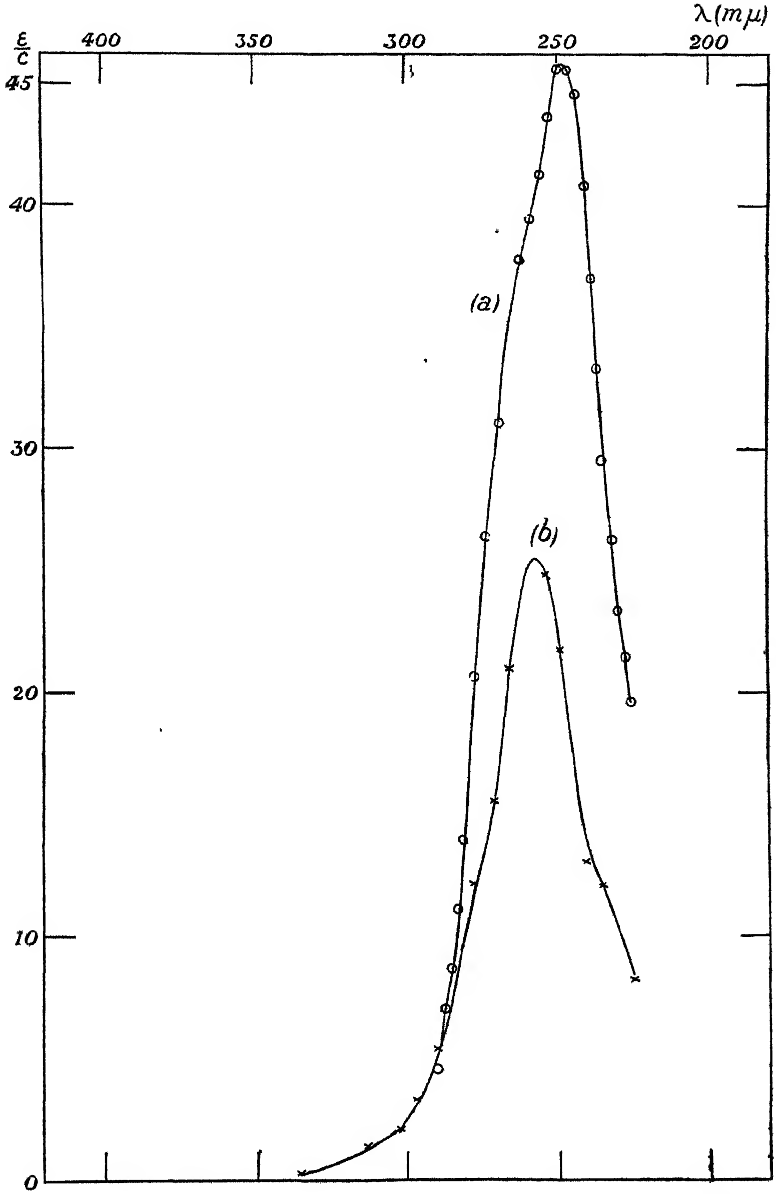

FIG. 1. Ultra-violet absorption curves of crystalline vitamin B_1, preparations (*a*) of Kinnersley, O'Brien and Peters, (*b*) of Windaus *et al.* Ordinates ε/c. Abscissæ mμ.

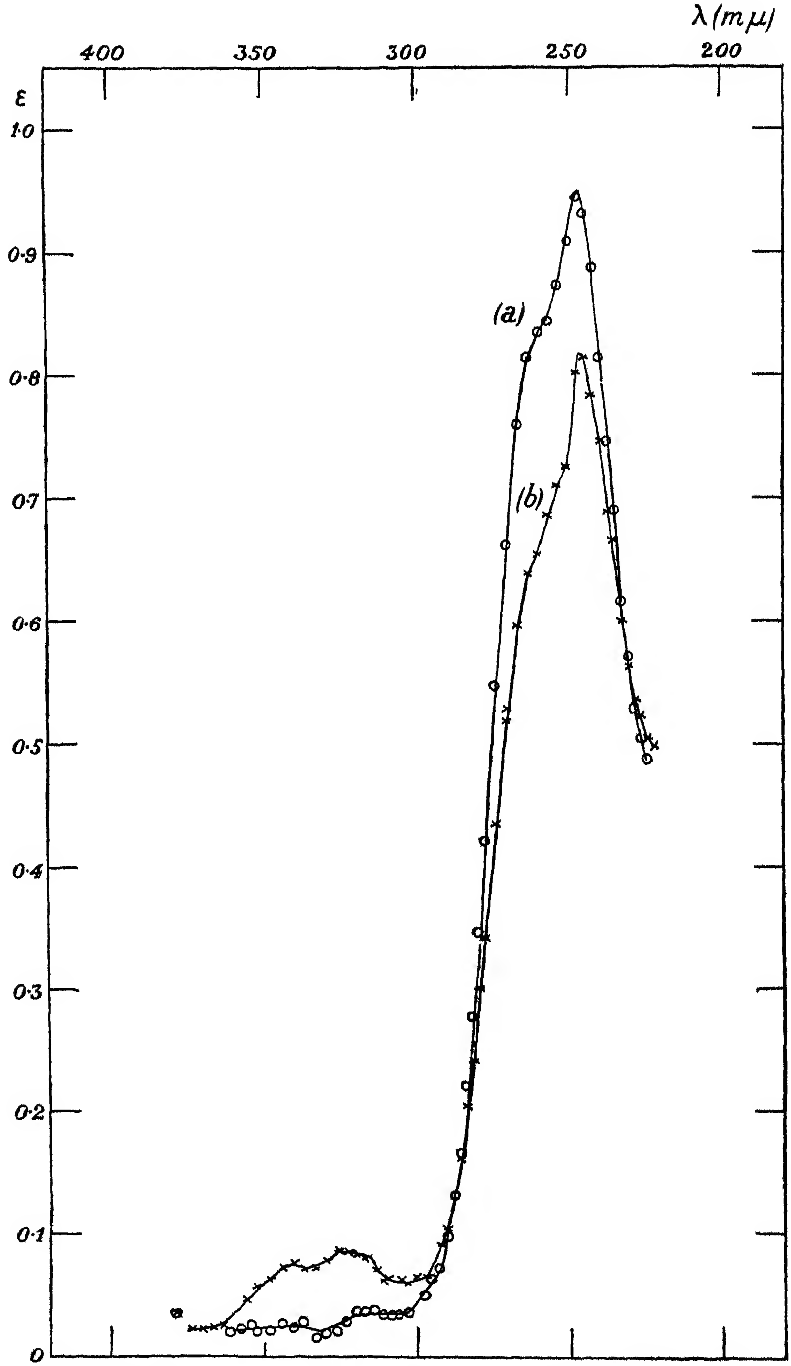

FIG. 2. Ultra-violet absorption curves in acid solution; (*a*) after slight alkaline treatment; (*b*) after more extensive alkali treatment. Ordinate ε. Abscissæ mμ.

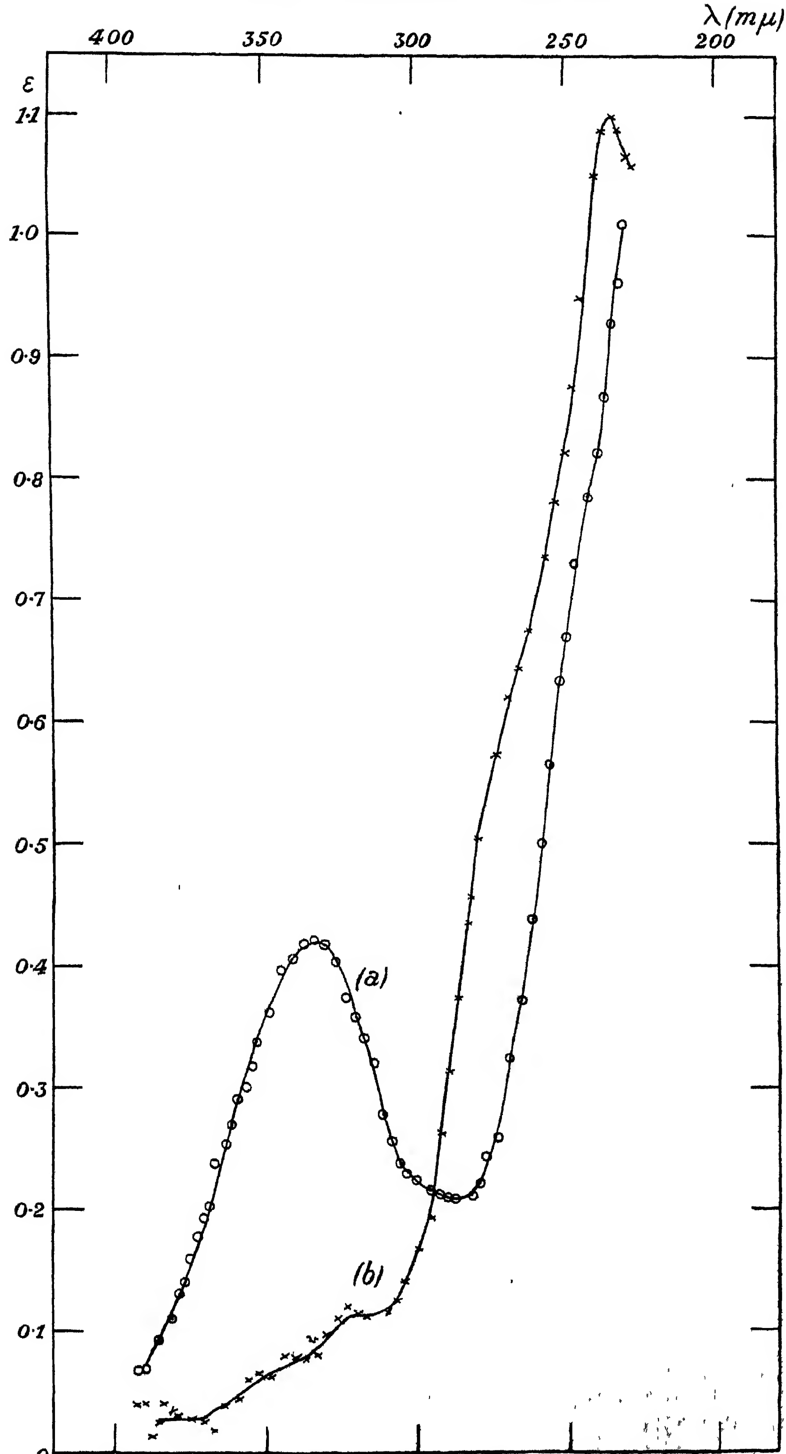

FIG. 3. Ultra-violet absorption curves of same solution as FIG. 2 (*a*) in N/10 NaOH.

Fig. 4 shows ε for :—

(*a*) Preparation 64 (19) made alkaline for the second time after passing through one complete cycle, showing that the 330 mμ band is still produced.

(*b*) The same after acidifying, giving the original absorption back again.

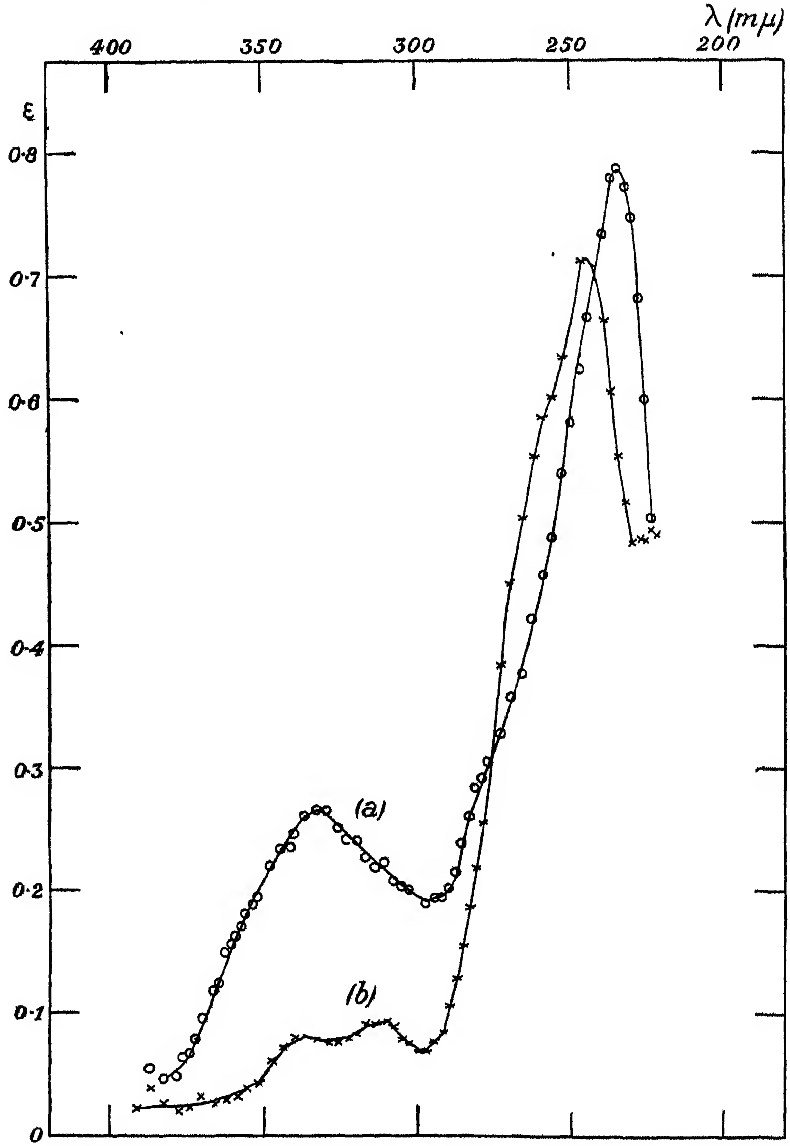

Fig. 4. Ultra-violet absorption curve for second treatment with alkali; (*a*) in NaOH solution; (*b*) in acid solution. Ordinate ε. Abscissæ mμ.

The lower intensity of the 330 mμ band in fig. 4 (*a*) as compared with fig. 3 (*a*) is not entirely due to dilution, though it may be accidental, as there is some evidence that the band is labile even at room temperature. The intensity in fig. 4 (*b*) can be accounted for by dilution. In figs. 3 and 4 the curves at wavelengths below 235 mμ are liable to considerable error since the absorption of the alkali itself rises very steeply. The existence of a maximum somewhere near 235 mμ in figs. 3 (*b*), 4 (*a*) and 4 (*b*) is, however, fairly certain.

Biological Activity.

The well-known inactivation of vitamin B_1 by alkali would suggest that the present treatment must reduce the activity substantially. Some tests performed (*a*) with the actual solutions used, and (*b*) with some similarly treated support this view. Previous to treatment, the activities were for 64 (19) $2 \cdot 2 \pm 0 \cdot 97 \gamma$ and for 61A (15) $1 \cdot 6 \pm 0 \cdot 46 \gamma$, curative day dose for pigeon (see Kinnersley, O'Brien and Peters, 1933).

(*a*) Upon the irradiated solution, we found for 64 (19) $3 \cdot 1 \gamma$ nil, $6 \cdot 9 \gamma$ 2·5 days, $8 \cdot 9 \gamma$ nil, and for 61A (15), $6 \cdot 1 \gamma$ nil, $12 \cdot 4 \gamma$ 2·5 days, $17 \cdot 8 \gamma$ nil. More of the solution was not available, but if we consider the tests as representing maximum and minimum variations, we get for approximate activity after treatment 61A (15), 11γ, and 64 (19), $5 \cdot 8 \gamma$, a diminution of some 55 per cent.

(*b*) Two tests upon the remainder of the same solution 64 (19) similarly treated with alkali + alcohol, but not irradiated, gave a similar answer, $22 \cdot 0 \gamma$ 4 days, $18 \cdot 3$ γ, 5 days, average $4 \cdot 6 \gamma$; there was again a diminution of some 50 per cent. Though more tests would be required to obtain an accurate estimate of the decrease, it is clear that there is more diminution in activity than in intensity of absorption. Hence the band at 245 mμ is not necessarily correlated with the activity.

Table I shows in column II the specific extinction coefficients, ε/C, of four preparations; one of which was less highly purified than the others. The preparation of Windaus *et al.* is also included for comparison. In column III are given the ratios of the extinction coefficient to the biological activity *a*, expressed in doses per cubic centimetre.

We wish to thank Dr. W. Herkel for doing some of the densitometric measurements, and Dr. E. R. Holiday for advice.

Table I.

Preparations.	Activity day dose/γ.	ϵ/C.	ϵ/a $\times 10^{-2}$
" Crude "	6·4	24·3	16·0
56 (8)	2·0	42·9	8·2
61A (15)	1·6	46·4	7·4
64 (19)	2·2	45 (approx.)	9·9
Windaus *et al.*	4·4	25 (at *ca.* 258 mμ)	11·0

Summary.

The highly potent crystalline preparations of vitamin B_1 made by Kinnersley, O'Brien and Peters show maximum ultra-violet absorption at 245–249 mμ. A substance is present which shows marked changes in absorption upon making alkaline; these changes are reversible.

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576 . 354 . 5 : 582 . 886 Oenothera.

Meiosis in Oenothera missouriensis.

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[Plates 1 and 2.]

Introduction.

A cytological study of the meiotic phenomena in *Oenothera* may not need an excuse in spite of the exhaustive studies of the genus made by numerous competent cytologists of this century. Up to the present time, all the investigators of *Oenothera* cytology have been successful in establishing that the basic (n) number of chromosomes in this genus is 7; although tetraploid (Gates, 1911), triploid (Catcheside, 1931), and trisomic numbers might occur either naturally (by mutation) or could be produced by experiment. It is also known that the somatic number of chromosomes corresponds with the number of chromosome bodies in the diakinesis and metaphase of the heterotypic division. Thus in diploid *Oenothera* species, hybrid, or mutant at the diakinesis of pollen mother cells 14 chromosomes have been shown to exist, without any doubt, in the configuration of a closed circle, in 7 ring pairs, or a mixture of free pairs and closed circles. Mathematically, there are 15 possible configurations in which 14 chromosomes can arrange themselves in the form of closed circle, ring pairs, or a combination of ring pairs and closed circles (Cleland and Blakeslee, 1931; Darlington, 1931). Of these 15 possible configurations 13 have already been reported in various *Oenothera* species, hybrids and mutants (Darlington, 1931). Regarding the origin and significance of these chromosome configurations investigators have not yet reached an agreed opinion. Apart from the genetical significance, the much disputed cytological question of parasynaptic and telosynaptic methods of chromosome pairing is yet far from a final solution. In *Oenothera* both the methods of pairing have strong supporters in consideration of observed cytological facts. The facts are (i) the continuous spireme (in leptotene stage); (ii) the pachynema and the diakinesis consisting of the 14 chromosomes arranged end to end. This arrangement, known as catenation of chromosomes, favours the telosynaptic rather than the parasynaptic union. Whereas (i) double threads at the prophase, (ii) the looping of the threads, and (iii) the half number of bodies (7 ring pairs)

at the diakinesis support the parasynaptic method of pairing of chromosomes. The occurrence of a complete catenation of 14 chromosomes in some *Oenotheras* and the presence of 7 free pairs in others naturally suggests the question—whether they can be correlated with the two methods of chromosome-pairing in the meiosis of *Oenothera.*

A detailed study of the meiosis in *Oenothera* species which show 7 free pairs in diakinesis is more likely to throw some light in understanding the actual situation regarding synapsis. Up to the present time, only four species of *Oenothera*, namely, *O. grandiflora* (Davis, 1909; Cleland, 1928; Illick, 1929), *O. Hookeri* (Schwemmle, 1924; Hoopener and Renner, 1929), *O. fransiscana* (Kulkarni, 1929), *O. purpurata* (Rudloff, 1929; Gates and Goodwin, 1931) have been reported to show 7 free pairs of chromosomes in diakinesis; of these only *O. Hookeri* is found wild. This paper is a study of essential stages of pollen meiosis in *O. missouriensis*, which shows 7 free pairs of chromosomes in diakinesis and heterotypic metaphase. This species has not been studied cytologically before. The existence of another species of *Oenothera* with 7 free pairs of chromosomes very much interested Professor R. R. Gates, F.R.S., when a preliminary study of the material was being made by me in his laboratory at King's College, London. Professor Gates kindly suggested to me at that time, that a detailed study of the meiosis of this species might throw much light in understanding the disputed points of *Oenothera* cytology. The work was begun in the laboratory of Professor Gates, but as I had to leave England in the midst of my work, it is now being completed in the laboratory of Professor S. P. Agharkar, University of Calcutta.

Material and Methods.

A few plants of *O. missouriensis* were being grown in the *Oenothera* cultures of Professor Gates at the Gardens of the Royal Botanical Society, Regents Park, London. *O. missouriensis* is of straggling habit and weak constitution. It is perennial and has large yellow flowers which are solitary at the axil of the leaf. The most characteristic part of the plant is the winged capsules. Owing to these striking peculiarities it is often placed in a separate genus Megapterium.

Young flower buds were collected and fixed in weaker Flemming's solution between the hours 2 to 4 p.m., as previously described (Hedayetullah, 1932). The succeeding processes were also the same. The microtome sections, both longitudinal and transverse, were cut from 16 μ to 18 μ in thickness. Newton's iodine gentian violet stain was used exclusively for the preparations, and gave a very satisfactory picture of all the essential stages.

Observations.

The archesporium consists generally of two, but occasionally of one longitudinal row of polygonal pollen mother cells. The polygonal shape of the cells at this stage must be due to the resultant action of crowding of a membranated distended fluid mass in a limited space bounded by the tapetum. The tapetal cells are as usual more densely stained than the pollen mother cells. The latter are filled with a fine reticulum of faintly stained chromatin threads and appear to be granular. In spite of elaborate search, nothing to corroborate Miss Leliveld's assertion of the occurrence of prochromosomes in *Oenothera* at this stage was found in the present material.

The reticulum condition of the nucleus is followed by the leptotene stage. In passing to this stage the pollen mother cells gradually lose their polygonal shape and become globular, but still they are attached tangentially with one another in the row. The nucleus in the leptotene stage is characterized here by a number of fine chromatin threads irregularly running across the nuclear cavity, fig. 1, Plate 1. As a rule, free ends are seen either in contact with the nuclear membrane or a little distance away from it. In some of the threads, an indication of folding or looping is clearly noticeable.

The occurrence of a single nucleolus is the rule in *O. missouriensis* ; there is an exception to this rule, as will be mentioned later. Unlike the observations made by Sheffield (1927) and others, the nucleolus is never organically connected at a point with the mass of chromatin threads in this or any later stages, although some of the threads may be in superficial touch with it in their course in the cavity of the nucleus. The position occupied by the nucleolus is always away from the nuclear membrane. This is a very extraordinary case in *Oenothera* cytology. In most of the sections studied it is only in the very early stage that the nucleolus is found to be away from the nuclear membrane, but with the progress of the prophase the nucleolus moves towards and attaches itself to the membrane, assuming a lenticular or crescent shape; it disappears in late diakinesis. In *O. missouriensis,* even up to the late diakinesis, the nucleolus maintains its position away from the nuclear membrane and never assumes a lenticular or crescent shape, figs. 1–2, Plate 1. The leptotene stage is of rather long duration, irregularly tortuous threads of the earlier leptotene gradually concentrate and bend round with one or more twists, fig. 2, Plate 1. The free ends of the folding threads are seen to approach each other, figs. 2 and 3, Plate 1, and ultimately fuse so as to form circular loops without free ends. These loops are distinctly less in number than the thin leptotene threads

They are next grouped together on one side of the nuclear cavity, fig. 4, Plate 1. This stage corresponds with the brochonema stage (Latter, 1926). A careful count at this stage and in the stage immediately following, fig. 5, Plate 1, has revealed that there are 7 such loops which correspond to the haploid number of the species. The brochonema stage was described by Latter (1926) in *Lathyrus*. There the brochonema appears "as a seven-spoked wheel." In *O. missouriensis* the loops forming the brochonema are more or less polarized (bouquet stage; Gelei, 1921; Kihara, 1927). That is to say, in *Lathyrus* "the hub" of the "spoked wheel" is central but in the present material it is eccentric. Latter (1932) has recently described other configurations at this stage in *Lathyrus*.

It will be realized from the above descriptions that no contraction of the chromatin threads into a knot (synizesis) is met with in the meiosis of the P.M.C. of *O. missouriensis*. This is also exceptional in this material and different from all other *Oenotheras* which have so far been observed. The period of synizesis has been regarded in this genus as the stage where very intimate relationship of the chromosomes is taking place. The result is that in the next open spireme stage a continuous spireme reveals itself in the nucleus. This spireme has been regarded as consisting of homologous paternal and maternal chromosomes alternately arranged (Farmer and Moore, 1905). In other words, the chromosomes are paired telosynaptically.* In the present material, although there is no evidence of the presence of either a synizesis or continuous spireme, yet it is clearly understood that between different chromosome threads union took place initially at one pair of free ends, the other pair of free ends still remaining unapproximated. The partially mated threads were then bent round and form loops so that the remaining pair of free ends might fuse together, resulting in a circular complete loop at the brochonema stage. A comparison of the stages depicted in figs. 1, 2, 3 and 4, Plate 1, will bear out the above statement. The method of synapsis involved will be discussed later.

The brochonema is followed by a stage where the loops are disentangled and spread out in the nucleus. In fig. 5A, Plate 1, 7 loops are marked out, and in fig. 5B, the same loops are shown separately. These loops are no doubt bivalent chromosomes. There are some characteristic features in the loops, which should be noted here. It has already been pointed out that the loops were formed by the union of the pairs of free ends of the chromosome threads. Now if this union is regarded as being established by "chiasma formations"

* The term acrosyndesis instead of telosynapsis or syndesis can be more fitly used, for the reason given by Percival, 1932 (p. 480).

(Darlington, 1929) between homologous parts of the chromosomes, then we see that in some loops these " chiasmata " are " terminalized " at both ends, forming a complete circular loop, figs. 5B, 6 and 7, in other cases the " chiasma" is terminalized at one end while it is " interstitial " at the other end, fig. 5B, 1, 3, 4 and 6 ; in still others the " chiasmata " are interstitial at both the ends, fig. 5, B2.

The loop bivalents are further enlarged and scatter themselves in the nucleus, fig. 6, Plate 1, the threads become thicker, and the loops undergo twist round their axis, figs. 7 and 8, Plate 1. It should also be noted that the synaptic union may disappear (dissolution of chiasmata) at one or both extremities. This brings out the appearance of secondary free ends of some of the chromosomes as seen in figs. 7–13, Plate 1. Where the arms of the bent round loops are very much approximated, accompanied by the dissolution of synaptic union (chiasma), there the chromosomes of a bivalent appear to lie side by side, one near the nucleolus, fig. 9, Plate 1, and one in the centre, fig. 10. This may be mistaken as an evidence of parasynapsis, but that cannot be true, because in the same nucleus complete loops can also be seen.

In fig. 11, Plate 1, there are less than 7 separate chromosome loops and in most of them the dissolution of synaptic union has taken place. The central big one must consist of more than one pair of chromosomes. This is evidently a loop of more than one pair of chromosomes, and this union among the chromosomes must be temporary and accidental; because in late diakinesis there are always 7 free pairs of chromosomes.

The number of " chiasmata " between different chromosome threads ranges from two to four, and a figure of 8 is also not an uncommon feature in some of the bivalents, persisting till late diakinesis in *O. missouriensis*, figs. 12, 15 and 20, Plate 1. The origin of the figure of 8 appears to be a simple one, namely, by a twist at or near the middle of a bivalent circle. The twist, however, is not a permanent feature, as in early metaphase no figure of 8 persists in any of the bivalent rings, fig. 23, Plate 1.

A few abnormalities are also observed during early diakinesis, some of which are represented in figs. 13, 14, 16 and 19, Plate 1. In fig. 13 some of the chromosome threads have not yet formed into complete loops although they have been thickened. It will be noted that in fig. 14 there are three univalent chromosomes, which are probably pieces of a bivalent loop. In figs. 16 and 17 several small nucleoli are seen, some of them in contact with the chromosomes. These small nucleoli arise by a process of budding; the actual process can be clearly seen in fig. 16, where two consecutive buds from a nucleolus are still

in contact. In the same figure, it will also be seen that a double satellite-like body is attached to one of the bivalents. In fig. 17 one of the chromosome rings is of larger diameter than the remaining ones. One bivalent in fig. 18 shows that the detached synaptic ends of the early stages are again approximating to re-establish the union. This nucleus shows a number of chromatic fragments besides the deeply stained nucleolus.

Interlocking of bivalents (Gates and Goodwin, 1931) is a very rare phenomenon in *O. missouriensis,* one of the very few cases being represented in fig. 19. There one bivalent is interlocked with two other bivalents, one of these again being interlocked with a third. The occurrence of interlocking of chromosome rings in *Oenothera* has been reported by various investigaors. Gates *et al.* (*loc. cit.*) have fully described the origin of interlocked chromosome pairs ; they came to the conclusion " that they are formed from the pairing and condensation of threads which were from the beginning separate from the threads belonging to other pairs." It has also been remarked in the same paper that the rings opened before the metaphase, as in that stage the ring pairs were rarely interlocked. That the rings open before the metaphase can be clearly testified from figs. 21 and 22, Plate 1, of the present material. Yet it must be pointed out that whether these opened rings are maintained or closed, is uncertain in *O. missouriensis.* In fig. 23, Plate 1, all the 7 pairs are rings, but in fig. 24, Plate 2, all of them are open. Openness in ring pairs (rod pairs) has been regarded as the condition of failure of chiasma formation by Darlington (1931) and Catcheside (1931). This question will be discussed later in the paper.

Figs. 25, 26 and 27, Plate 2, show the multipolar conditions of the nucleus. In fig. 25 there is a big ring which resembles somewhat a figure of eight. It is more likely that this figure of eight is due to a very close approximation of two rings. This is more probable, because otherwise the nucleus would have less than 7 bivalents. In fig. 26 three rings are interlocked like links in an iron chain, and there is also an open ring (rod pair). Fig. 27 shows that the open ring is further stretched in a more or less zigzag fashion.

The multipolar spindle then becomes bipolar. Figs. 28 to 36, Plate 2, show various successive stages of spindle arrangements of chromosomes. Till before the beginning of anaphase of the heterotypic division the bivalents occupy positions at different planes of the spindle. In fig. 28 all except one are in one plane, whereas in figs. 29 and 31, Plate 2, almost every bivalent has its own plane. In some cases the rings open and form rod pairs ; this is evident from figs. 31, 32, 33 and 34, Plate 2. One cannot be very sure from such figures whether the mates of these rod pairs would separate in the

anaphase; but from the telophase count of the heterotypic division, a regular distribution of 7 chromosomes has been observed unexceptionally. This ultimately proves, that if irregular separation of chromosomes ever takes place in heterotypic division it must be rare.

The case represented in fig. 35, Plate 2, shows that the chromosomes do not arrange themselves in zig-zag fashion. This should be expected in the present material, because zig-zag arrangement of the chromosomes in the metaphase is a rule in catenation-forming *Oenothera* (Cleland, 1925). It will be seen in the figure, that here the chromosome rings are situated parallel with one another and at right angles to the equatorial plate. The attachment of the spindle fibre is also clearly seen at this stage at the middle of each chromosome.

Regular separation of the bivalents takes place during the anaphase of the heterotypic division, thus bringing about the reduction of the chromosome complements. Fig. 36 is a drawing from a middle anaphase, where 7 chromosomes are clearly seen passing towards each pole.

In telophase of the heterotypic division, the chromosomes become rounder, fig. 37, Plate 2. This stage is of much shorter duration. As a rule, chromosomes do not clump together at this stage in *O. missouriensis*, they remain separated and undergo simple division, fig. 38, Plate 2. It is remarkable that Maltese cross-like or charged gold leaf-like structure of chromosomes so characteristic of interphase of other *Oenotheras* (Gates, 1928) is conspicuously absent in *O. missouriensis*. In fig. 38 the chromosomes of the daughter nuclei are undergoing homotypic division. In one nucleus the side view of the spindle at the equatorial stage is seen. The other nucleus is also probably at the same stage, but is seen in polar view.

Discussion.

Weismann's theoretical prediction of halving of nuclear material of the ancestral germplasm on the practical consideration of the phenomenon of fertilization has long been materialized in the discovery of the reduction of chromosome numbers (nuclear elements) during the heterotypic divisions of innumerable organisms belonging both to the animal and plant kingdoms. Although a reduction in the number of chromosomes was found to take place in the first division, yet another, second, division of the daughter nuclei immediately followed. The sum-total of the processes of these two contiguous divisions is known as meiosis, as distinguished from mitosis or somatic division of the vegetative nucleus. In meiosis, before the reduction of chromosomes, it has been shown that their pairing at the metaphase of the heterotypic

division, forming the haploid number of chromosome pairs, is involved. This pairing condition of the chromosomes at the metaphase and diakinesis, coupled with the re-discovery of Mendel's laws of segregation of factors in the progeny of a cross, stimulated the study of meiosis in both animal and plant material. The detailed and critical study of the different phases of meiosis went to prove that the reduction of chromosomes necessitated a previous synapsis (pairing) among the chromosomes of paternal and maternal origin. Therefore, the phenomena of fertilization, synapsis, and reduction became a co-ordinated process in the physiology of chromosome behaviour, at least in the life cycle of the sexually evolved organisms.

The relation of synapsis with reduction depends entirely on fertilization so far as the physiological aspect of the chromosomes is concerned. That is to say, the paternal and maternal chromosomal elements which come together in the fertilization of the egg and form the composite nucleus of the new generation, again become intimately associated in the synapsis of meiosis before their separation takes place in the reduction of chromosomes. Synapsis, therefore, is the time and occasion when the actual conjugation from the genic point of view between the paternal and maternal elements takes place. The cytological question of synapsis in meiosis became all the more important with the gaining of ground by the chromosome theory of inheritance of Morgan; because interchange of genes is only possible when different chromosomal elements become intimately associated in synapsis at meiosis.

Attention was drawn to the modes of synapsis to solve the question of reduction, *i.e.*, how the half number of chromosome bodies arose at the metaphase from a prophase of meiosis, which in mitosis gave rise to the full somatic number. It was believed then, that chromosomal elements in synapsis joined end to end (telosynapsis) so as to form a continuous spireme, which by segmentation gave rise to the half or whole number in the metaphase of meiosis and mitosis respectively (Farmer and Moore, 1905).

The occurrence of double threads in meiosis was described by other writers as a synapsis of two single chromosome threads resulting in their pairing side by side (parasynapsis) (Gregoire, 1910). The doubleness was also regarded as incompatible with telosynapsis. This incompatibility was, however, shown to be fallacious by the critical observations of Digby (1919). She was able to show that the doubleness was due to the persistence of the split halves of single chromosomes continuing from the premeiotic division. The supporters of parasynapsis came again with observational facts of the appearance of four threads side by side at the later stages (diplotene) of meiosis as a

redoubtable support for the parasynaptic modes of synapsis and an unsurmountable obstacle to the telosynaptic mode of synapsis. The obstacle was, however, overcome by Farmer and Moore (*loc. cit.*), who were able to show convincingly that the appearance of four threads side by side must arise from a secondary side by side pairing of chromosomes originally united end to end. It has also been pointed out recently (Hedayetullah, 1931, 1932) that the quadruple structure of the chromosomes at the diplotene may also be explained on the basis of the chromonema theory of chromosome structure, and at the same time it is compatible with the telosynaptic mode of synapsis, as well as with parasynapsis. In short, the literature abounds with the description of both the methods of synapsis by competent observers; and the most interesting part of it is, that often in the same object both the modes of synapsis have been described. *Oenothera* is one among many in which both the modes of synapsis have been supposed to occur (see Leliveld, 1931).

It will be superfluous to discuss here the work of various cytologists regarding the methods of pairing of chromosomes in *Oenothera* when this has already been dealt with by Leliveld. It may be said that the majority of them considered the mode of synapsis as telosynapsis, while some of the recent authors (Darlington and Catcheside, *loc. cit.*) attempt to prove that the mode of synapsis is parasynapsis on the basis of the chiasma theory of chromosome pairing. According to them, chiasmata result from the association of the chromatids differently at different points with exchange of partners. This association takes place late in the prophase of meiosis at the diplotene stage. Although Darlington asserts that the method of chromosome pairing in *Oenothera* is parasynaptic, yet he considers the prophase of meiosis in *Oenothera* to be unintelligible. But it is a big step from an unintelligible prophase to the establishment of parasynapsis in *Oenothera* on the assumption of formation of chiasmata, and their terminalization resulting in the configuration of the chromosomes at diakinesis and metaphase.

Both the supporters of parasynapsis and telosynapsis theories in *Oenothera* have found the main plank of their support in the chromosome configurations in diakinesis and metaphase. Nobody can deny that here the real association among the chromosome bodies is at their extremities, whether it is a ring of two chromosomes or more. In other words, the association of chromosomes at the said stages does not even resemble in the slightest degree what is conveyed by parasynapsis. Accordingly telosynapsis (better telosyndesis) has been regarded as the mode of union among the chromosomes of *Oenothera* in establishing the relation between the method of chromosome pairing and their

configurations at diakinesis and metaphase. It is evident, therefore, that the supporters of telosynapsis assume that the affinity for union lies in the extremities of the chromosome bodies, consequently chromosomes are seen to be united by their ends only. It has been pointed out in the introduction that the 14 chromosomes of *Oenothera* are capable of forming mathematically 15 configurations in the form of rings or chains among themselves, and of these 13 configurations have already been reported. That is to say, the chromosome configurations in *Oenothera* are confined to the form of rings and chains, and nothing else, showing the existence of a peculiar organization among the chromosomes on the basis of affinity at the ends; whereas the recent work of Hoare (1931, 1932) in the genus *Hypericum* has brought to our knowledge, that out of a number of species only one shows exact configuration (complete ring) like some *Oenothera* chromosomes. Again, Percival (1932) in a number of hybrids of wheat and ægilops finds the chromosomes associated parasynaptically or telosynaptically in the same nucleus. He has come to the conclusion from his studies that the affinities for union between chromosomes are of varying degree. When the affinity is great (*i.e.*, distributed over the whole length of the chromosomes) the union is parasynaptic; and when the affinity is less (*i.e.*, localized at a point), the union is telosynaptic (acrosyndesis). Hence the two methods of pairing differ only in degree and not in kind.

Darlington's enthusiasm for establishing the universality of parasynapsis in meiosis has led him to a curious contradiction. A ring is formed in *Oenothera*, according to him, by formation of chiasmata at random in the paired segments (1929, 1931, *a*, *b*) and then by their terminalization following segmental interchange, so that at diakinesis the fine connections between chromosomes represent the chiasmata. Formation of chiasmata and their terminalization necessarily implies an organized movement of chiasmata. He also states that chiasmata form only between homologous portions of the chromatids. It follows from his statement that the remaining portions of the chromatids of pairing chromosomes where no chiasmata are formed, are non-homologous. Darlington contradicts himself when he says that a movement of the chiasmata takes place to bring about their terminalization, because he suggests in one place (1913, *b*, p. 422) that an arrest of movement of chiasmata occurs owing to a change of homology, *i.e.*, chiasmata cannot proceed through the non-homologous region of the chromosomes. The movement of chiasmata must be arrested at the points where they are formed. Therefore chiasmata have simultaneously fixed their position with their formation. Hence it is clear that Darlington's

theory of ring formation by terminalization of chiasmata is untenable according to his own hypothesis.

Again one may legitimately ask how would 14 chromosome threads associate parasynaptically in early prophase so that 14 chiasmata would form among them, and their terminalization bring out the complete ring condition in diakinesis? As already mentioned, Darlington expressed his inability to understand the prophase stage in *Oenothera*. Catcheside (1931, p. 175, fig. 5, A, B, C, D), however, gives diagrammatical drawings to substantiate the method of association of chromosomes prior to, during, and after the formation of chiasmata. Even in these diagrammatic drawings one would see that in no stage are a pair of chromosomes wholly associated with each other side by side. Here, too, one sees only a partial association between the mates of a pair, and the final association at the extremities only. It is evident, therefore, that the methods of synapsis asserted by Darlington and Catcheside in *Oenothera* differ only in degree but not in kind.

It has already been pointed out in the description of the present material how the chromosome threads associate at their ends, showing that affinity for union lies at the extremities of the pairing mates. It may be advantageously explained by the help of diagrams below, how bivalent rings are formed in the present case on the basis of affinity for union existing only at the extremities of the pairing chromosomes. AB and CD are two pairing chromosomes,

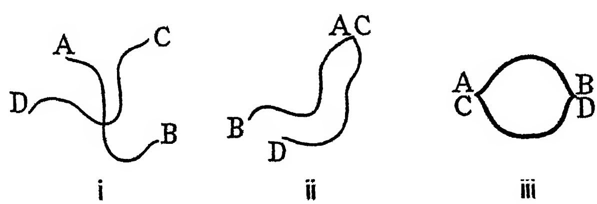

FIG. 40.—(i) AB and CD are two pairing chromosomes. (ii) During synapsis the ends A and C meet first. (iii) Next B and D meet, giving a ring-like form of the bivalent pair.

fig. 40, having affinity for union at the extremities A, B, C, D. In synapsis the extremities A and C unite first and then B and D, giving a ring-like form of the bivalent pair.

In conclusion I take the opportunity of expressing my heartfelt thanks to Professor Gates for his continuous help and encouragement. My thanks are also due to Professor S. P. Agharkar and the Council of Post-Graduate

teaching in Science of the University of Calcutta for giving me facilities to continue my work in the Biological Laboratories of the University.

Summary.

(1) The meiosis in pollen mother-cells of *O. missouriensis* is studied for the first time.

(2) The leptotene is characterized by a number of irregularly running fine chromatin threads in the nucleus.

(3) The threads of the earlier leptotene gradually concentrate and bend round.

(4) The free ends of the folding threads, which are distinctly less in number (following union between threads at one end first) than early leptotene stage, approach each other. They ultimately fuse and form circular loops.

(5) Seven such loops are formed which give rise to seven free bivalent ring pairs in diakinesis.

(6) The method of synapsis involved, is described as acrosyndesis (telosynapsis), depending on affinity of the chromomeres lying at the extremities of the chromosomes.

(7) The question of para- and telo- synapsis is discussed and it is shown that the two methods of chromosome conjugation vary only in degree, and not in kind.

(8) The nucleolus never attaches itself to the nuclear membrane, and certain irregularities are seen in its behaviour.

(9) The reduction division of the chromosomes is normal and regular, and no non-disjunction of the chromosome pairs was observed.

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EXPLANATION OF PLATES.

All figures are sketched at table level with the aid of a camera lucida. A 2 mm. Zeiss aprochromatic objective (N.A. 1·25) and Zeiss oc. × 15 were employed for all drawings. The drawings have been reproduced without reduction. Magnification × 2000. The figures represent either entire pollen mother-cells of *O. missouriensis* or their nuclei.

PLATE 1.

FIG. 1.—P.M.C. nucleus in leptotene stage showing fine irregularly running chromatin threads. Note the free ends are away from the nuclear membrane.

FIG. 2.—Nucleus showing the concentration of the leptotene threads and their bending round with one or more twists. Note the free ends are less in number than in the previous stage.

FIG. 3.—The same as above, but of little later stage. Note the approach of the free ends of each thread which ultimately fuse and form circular loops. The loops are distinctly less in number than the leptotene threads.

FIG. 4.—Nucleus showing the grouping together of the circular loops (brochonema stage). Mark the eccentric position of the "hub."

FIG, 5, A.—Nucleus of later stage than above, showing disentanglement of the loops. There are seven such loops (marked by numbers) corresponding to seven bivalent ring pairs.

FIG. 5, B.—The loops of the above nucleus are shown separately.

FIG. 6.—Nucleus showing further enlargement and scattering of the loops in the nuclear cavity.

FIGS. 7 and 8.—A little later than above, showing the twist round of the loops.

FIGS. 9 and 10.—Early diakinesis. The dissolution of synaptic union is taking place in some of the loops.

FIG. 11.—Nucleus showing less than seven pieces of loops, most of which have lost their synaptic union. The central big one must be composed of more than one pair of chromosomes.

FIG. 12.—Early diakinesis. Chiasmata are formed in the bivalent pairs.

FIG. 13.—Early diakinesis. Some of the chromosome threads have not yet formed loops.

FIG. 14.—Diakinesis. Three univalents are seen.

FIG. 15.—Diakinesis. One of the bivalents having the shape of a figure of eight.

FIG. 16.—Diakinesis showing several small nucleoli, one in state of budding. Note the satellite-like attachment to one of the bivalents.

FIG. 17.—Diakinesis. A chromosome ring of peculiarly large diameter is seen.

FIG. 18.—Diakinesis. The re-approach of the detached synaptic ends is seen in one of the bivalents.

FIG. 19.—Diakinesis, showing interlockings among some of the ring bivalents.

FIG. 20.—Diakinesis. The persistence of the shape of figure of eight is seen in one of the bivalents till late.

FIGS. 21 and 22.—Diakinesis, showing cases of opening of the ring pairs before metaphase.

FIG. 23.—Very late diakinesis showing seven ring pairs.

PLATE 2.

FIG. 24.—The same as fig. 23 but here all the pairs are rod pairs (opened rings).

FIGS. 25 to 27.—Showing multipolar condition of the nuclei. In fig. 25 one ring has the form of figure of eight. Fig. 27 shows an opened ring stretched in a zig-zag manner.

FIG. 28.—Bipolar spindle. All bivalents except one arranged in one plane.

FIGS. 29 to 31.—Bipolar spindles. Each bivalent situated in a different plane.

FIGS. 32 to 34.—Spindles showing several rod pairs.

FIG. 35.—Metaphase spindle. Chromosome rings situated parallel with one another and at right angles to the equatorial plate.

FIG. 36.—Spindle in mid-anaphase. Note seven chromosomes (univalent) passing to each pole.

FIG. 37.—P.M.C. in heterotypic telophase. Seven chromosomes (univalent) seen in each newly forming nucleus.

FIG. 38.—P.M.C. showing homotypic division in the daughter nuclei, one in side view and the other in polar view.

FIG. 39.—A tetrad.


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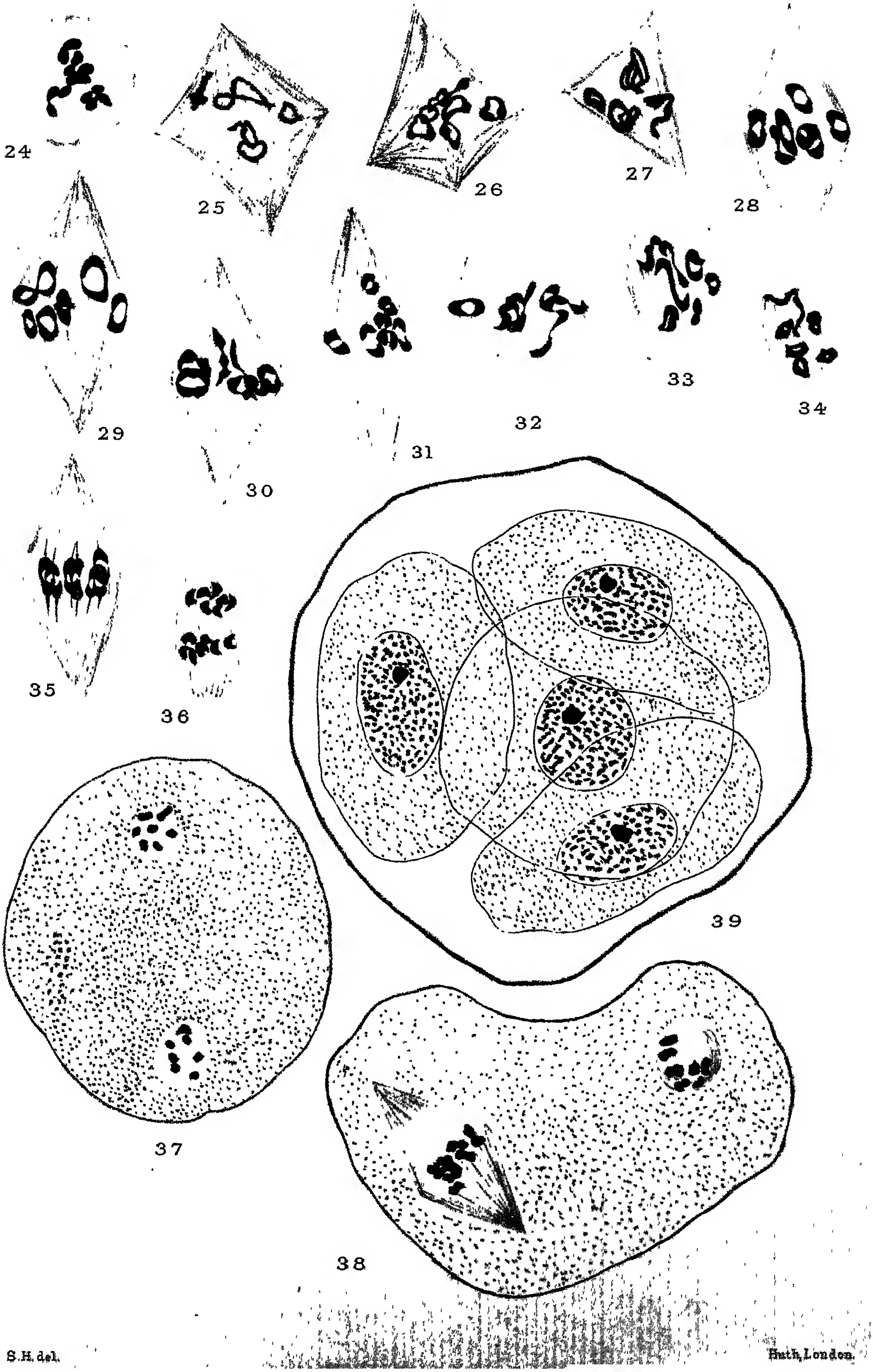

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Studies on Antagonism. II.—*The Effect of Previous Immersion of Potato Tuber Tissue in Solutions of Monovalent, Divalent, and Trivalent Kations on the Subsequent Absorption of the Ammonium Ion.*

By Geoffrey F. Asprey.

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Introduction.

In the previous paper of this series (Asprey, 1933) it was suggested that the antagonistic action of a kation towards an accompanying kation depended on two factors; one of these is its competition with the accompanying kation This factor will always tend to reduce the absorption of both ions as compared with that taking place from their pure solutions. The second factor is the specific effect of the ion on the absorptive power of the tissue; this was presumed to differ with the valency of the kation. It was supposed that monovalent kations tend to increase, and divalent, and trivalent kations to decrease, the intake of other kations by the tissue. In the experiments previously described the antagonistic action exhibited by various kations towards the ammonium ion in its absorption by potato tissue was observed. It was found, however, that only one monovalent salt, lithium chloride, actually increased the ammonium intake. It was shown that the apparent divergence in the behaviour of lithium and the other monovalent salts could be explained by assuming that the specific effect of all of them on the tissue was the same; to increase the absorption of ammonium, but that the effect of sodium and potassium was very much less than that of lithium. Reduction in absorption caused by ionic competition would then mask the relatively weak tendency of sodium and potassium to increase the intake of ammonium.

Although this provided a suitable explanation of the results obtained, there was at that time no evidence either to support or to disprove it. It was decided that some light would be thrown on this matter if experiments could be devised in which the specific effects of salts on the tissue alone were determined. It was thought that by immersing the disks of potato tuber first in solutions of the salts under consideration for a known period and then in ammonium chloride, information might be obtained about the specific effect of these salts on ammonium intake, since ionic competition would be eliminated.

Methods.

Disks of potato tuber 2 cm. in diameter and 1 mm. thick were obtained and washed as previously described (Asprey, 1933). Batches of 80 were then taken, dried between filter paper, and immersed for 2 hours in 200 c.c. of the salt the action of which was to be studied. During this time the disks were placed in bottles in the shaking apparatus which was maintained at a temperature of 25° $\pm$ 0·5° C. After this period they were removed from the solution, dried quickly between filter paper, placed in 200 c.c. of a 0·02 N solution of ammonium chloride and returned to the shaker for various known periods up to 24 hours. The percentage absorption of ammonium was then determined. In all cases control experiments were performed using distilled water instead of the salt solutions. All experiments were conducted in triplicate.

The salts employed were chlorides of sodium, potassium, lithium, calcium, magnesium, and aluminium. Preliminary experiments had shown that with 0·02 N solutions of the salts the effects produced were similar to, although less marked than, those obtained with 0·10 N solutions. As definite results were required the stronger concentration was employed throughout.

The primary object of this work was a comparison of the effect of the various monovalent salts used. Accordingly experiments with these salts were always made at the same time with material from the same sample of tissue and placed for the same periods in ammonium chloride. This also applies to experiments conducted with aluminium, calcium, and magnesium chlorides.

Ammonium was estimated, as before, by means of a micro-kjeldahl apparatus. All solutions were standardized with silver nitrate and all chemicals were analytical reagents obtained from British Drug Houses, Ltd.

Results.

In Table I are given the results of a series of experiments in which tissue was immersed for different periods up to 24 hours in each of the three monovalent salts employed and in distilled water before subsequent introduction to 0·02 N ammonium chloride. From an examination of this table it will at once be evident that, as previously suggested (Asprey, 1933), these salts cause an increase in the absorption of ammonium as compared with the control. This is perhaps seen more clearly in fig. 1. Furthermore, it will also be apparent as was previously assumed, that potassium and sodium bring about much less increase in subsequent ammonium absorption than does lithium. The increase

Table I.—The effect of previous immersion of disks ot potato tuber in 0·10 N solutions of lithium, sodium, and potassium chlorides on the subsequent absorption of the ammonium ion from a 0·02 N solution of ammonium chloride.

	Percentage absorption of ammonium.							
	Experiment 1 (1 hour).				Experiment 2 (2 hours).			
				Mean				Mean
Distilled water ..	6·2	6·6	6·4	6·4	8·8	8·7	9·2	8·9
KCl	7·6	8·0	8·1	7·9	10·5	10·2	10·1	10·3
NaCl	8·7	8·5	8·6	8·6	10·0	11·0	11·2	10·7
LiCl	13·0	13·3	13·9	13·4	15·9	16·5	17·1	16·5
	Experiment 3 (3 hours).				Experiment 4 (5 hours).			
				Mean				Mean
Distilled water ..	9·8	9·9	10·2	10·0	12·4	12·4	12·2	12·3
KCl..	13·1	13·1	13·6	13·3	15·1	15·1	14·8	15·0
NaCl	13·0	13·3	13·6	13·3	15·6	15·2	15·4	15·4
LiCl	24·5	24·5	25·2	24·7	30·4	29·3	30·6	30·1
	Experiment 5 (7 hours).				Experiment 6 (24 hours).			
				Mean				Mean
Distilled water .. .	13·6	13·4	13·65	13·55	18·7	18·0	18·0	18·2
KCl....	16·0	17·5	16·4	16·6	22·0	21·5	22·8	22·1
NaCl	17·3	17·3	17·6	17·4	22·8	23·0	22·3	22·7
LiCl	31·5	31·7	31·2	31·5	35·6	39·0	38·7	37·8

caused by potassium is always slightly less than that brought about by sodium. This will be shown more clearly later (Table III).

It was noticed that after treatment with lithium there is much more visible exosmosis from the tissue into the ammonium chloride solution than with any of the other salts employed. Attention has already been drawn to this property of lithium in the first paper of this series. In a similar series of experiments to the above the two divalent salts calcium and magnesium chlorides and the trivalent salt aluminium chloride were used. Results obtained may be seen in Table II and, expressed graphically, in fig. 2, from a consideration of which it will at once be evident that these three salts all decrease the subsequent intake of ammonium by the tissue. They thus have an effect directly opposite to that of the monovalent salts. The observed reduction in

ammonium intake brought about by magnesium is very slight, sometimes quite within the limits of experimental error, and it causes at all times much less reduction of absorption than does calcium chloride. Treatment of the

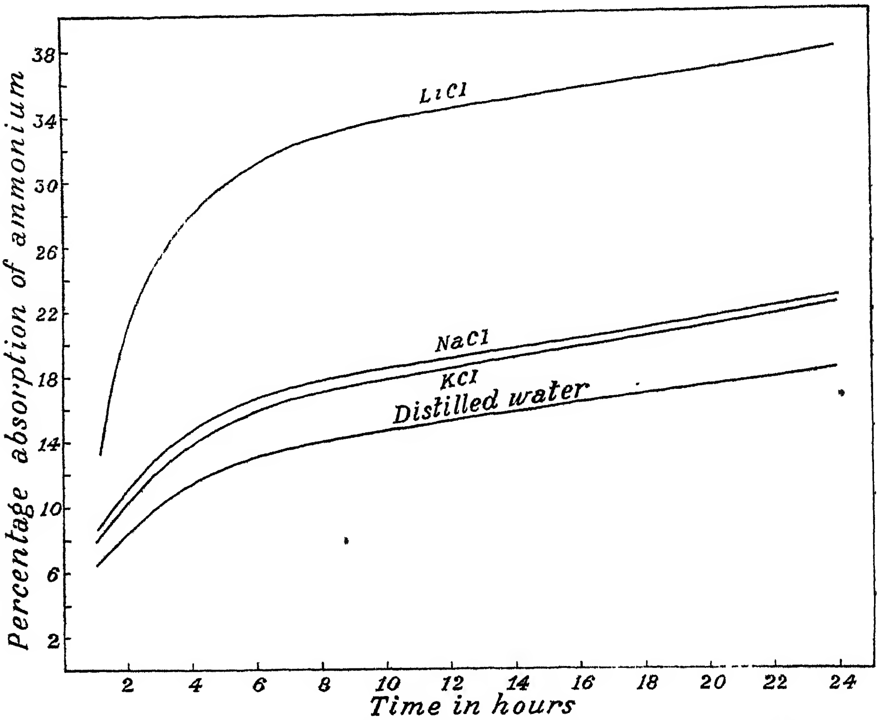

FIG. 1.—The effect of previous immersion of disks of potato tuber tissue in 0·10 N solutions of lithium, sodium, and potassium chlorides on the subsequent absorption of the ammonium ion from a 0·02 N solution of ammonium chloride.

Table II.—The effect of the previous immersion of disks of potato tuber in 0·10 N solutions of magnesium, calcium, and aluminium chlorides on the subsequent absorption of the ammonium ion from a 0·02 N solution of ammonium chloride.

	Percentage absorption of ammonium.							
	Experiment 1 (1 hour).				Experiment 2 (3 hours).			
				Mean				Mean
Distilled water ...	6·0	6·0	6·0	6·0	9·5	9·6	9·8	9·6
$MgCl_2$	5·5	5·5	5·8	5·6	9·4	9·4	9·4	9·4
$CaCl_2$	4·6	4·4	4·3	4·4	7·8	7·8	7·6	7·7
$AlCl_3$	2·6	2·6	3·1	2·8	5·5	5·7	5·6	5·6

Table II—(continued).

	Experiment 3 (6 hours).				Experiment 4 (9 hours).			
				Mean				Mean
Distilled water . .	15·7	15·0	15·5	15·4	16·9	17·2	17·6	17·2
$MgCl_2$	13·4	13·6	14·4	13·8	15·7	16·1	16·9	16·2
$CaCl_2$	11·6	11·3	12·0	11·6	13·0	12·7	13·0	12·9
$AlCl_3$	8·6	8·5	8·2	8·4	9·4	10·1	10·6	10·0

	Experiment 5 (24 hours).			
				Mean
Distilled water .	20·0	19·5	19·3	19·6
$MgCl_2$	19·6	18·9	19·1	19·2
$CaCl_2$	17·2	17·2	16·0	16·8
$AlCl_3$	12·6	13·2	13·1	13·0

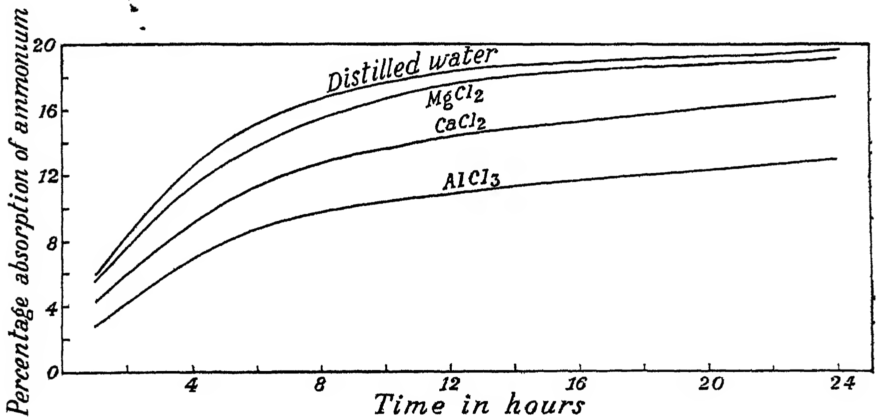

FIG. 2.—The effect of previous immersion of disks of potato tuber tissue in 0·10 N solutions of magnesium, calcium, and aluminium chlorides on the subsequent absorption of the ammonium ion from a 0·02 N solution of ammonium chloride.

tissue with aluminium chloride results in a very marked decrease in ammonium absorption, much more than with either calcium or magnesium chlorides.

It is noteworthy that all the salts used, whether they increase or decrease the subsequent intake of ammonium by the tissue, do so to an extent that is remarkably constant, at least up to 24 hours. This fact is perhaps more easily realized when the increase or decrease caused is calculated for the various experiments performed on the basis that absorption for the control experiment is 100. This has been done using the mean values given in Tables I and II and may be seen in Table III. The figures given, which are calculated to the

Table III.—The absorption of ammonium by potato tuber tissue from 0·02 N ammonium chloride after 2 hours immersion in the solutions indicated. (Absorption after previous immersion in distilled water is taken as 100.)

Time in hours.	LiCl.	NaCl.	KCl.	$MgCl_2$.	$CaCl_2$.	$AlCl_3$.
1	209	136	123	93	73	47
2	185	120	116	—	—	—
3	247	133	133	98	80	58
5	245	125	122	—	—	—
6	—	—	—	90	75	55
7	232	128	122	—	—	—
9	—	—	—	94	75	58
24	208	125	121	98	86	66
Mean	221	128	123	95	78	57

nearest whole number, must be considered as only approximate; nevertheless, considering the method of their derivation, it is particularly noteworthy that up to and including the 24-hour period the reduction or increase in ammonium absorption should be so constant. Knowing these mean values it is possible, if we know the absorption of ammonium for all periods up to 24 hours by untreated tissue, to predict, approximately, for any given time up to 24 hours, what the absorption would have been if the tissue had been previously immersed in any of the above salts. Thus, if the effect of treatment with 0·10 N lithium or calcium chloride were required, we should have to multiply the known value by 2·21 or 0·78 respectively. This applies similarly to the other salts. For the sake of simplicity later it is proposed to call these multiplying factors "Ammonium absorption numbers." The ammonium absorption numbers of the various salts employed in a concentration of 0·10 are then:—

LiCl ..	2·21	NaCl ..	1·28	KCl ..	1·23
$MgCl_2$ ..	0·95	$CaCl_2$ ..	0·78	$AlCl_3$ ..	0·57

Having obtained some quantitative data as regards the specific effect of salts on the tissue, it is interesting to compare them with data yielded by experiments where both postulated factors are in operation. There is given in Table IV the average intake of ammonium ion from 0·02 N ammonium chloride in the presence of 0·10 N solutions of the salts under consideration. The figures have been calculated from the graphs constructed from the data given in a previous paper (Asprey, 1933) and in a similar way to those in Table III; that is, the absorption which has taken place from the pure solution of ammonium chloride for the same time interval is taken as 100. Data given for alumin-

ium are only for periods up to 10 hours, as after this, it will be remembered, the absorbed ammonium was apparently returned to the external solution. The figures given for magnesium chloride are for ammonium intake in the presence of the 0·02 N solution, as experiments with 0·10 N were impracticable. For various reasons data for experiments of less than 2 hours duration are not given. During the preliminary periods of immersion there is not only the possibility that adsorption is taking place on the external cell walls of the tissue but the time necessary for the salts to reach and react with the absorbing cells themselves must be taken into consideration. Both these factors would prevent the expression of the real antagonistic action of the salts towards ammonium chloride in its absorption by the disks of tissue. Even after 2 hours' immersion it is quite possible that these factors are not entirely negligible. Furthermore, for small periods of immersion a very slight experimental error will be magnified when the antagonistic action of the salts is expressed in the manner shown, and even for the periods given the figures must be considered as only approximate. Nevertheless, it will be evident that for each salt they are fairly constant.

Table IV.—The absorption of ammonium from solutions containing 0·02 N ammonium chloride and the respective salts indicated in a concentration of 0·10 N. (Absorption from a pure 0·02 N solution of ammonium chloride is taken as 100.)

Time in hours.	LiCl.	NaCl.	KCl.	$MgCl_2$ (0·02 N).	$CaCl_2$.	$AlCl_3$.
2	114	81	73	62	52	23
5	120	78	70	71	50	18
10	119	73	65	71	47	16
24	122	71	63	71	50	—
Mean	119	76	68	69	50	19

From a comparison of the mean values given in Table IV with those of Table III it will be noticed that in all experiments less absorption of ammonium takes place from the mixed solution of salt and ammonium chloride than that taking place from a pure solution of ammonium chloride after previous treatment of the tissue with the other salt. As in the earlier study, although from different evidence, one is again forced to the conclusion that the mere presence of the salt in the ammonium chloride solution, quite apart from its ammonium absorption number, results in a reduction of intake of ammonium by the tissue.

This fact is perhaps most striking with the two monovalent salts sodium and potassium chlorides. These two salts in a concentration of 0·10 N have ammonium absorption numbers of 1·28 and 1·23 respectively, but when introduced into the ammonium chloride solution actually reduce its absorption by 24 per cent. and 32 per cent. respectively. Again, results show that previous immersion of the disks in 0·10 N magnesium chloride does not reduce subsequent intake of ammonium nearly so much as the presence of 0·02 N magnesium chloride in the ammonium chloride solution.

Discussion.

The experiments described in this paper support the suggestion that the antagonistic action of one kation towards another depends on two factors, namely (1) its specific effect on the absorptive capacity of the tissue for that ion, and (2) a factor which in all cases tends to reduce its intake. This latter factor, as previously suggested, is possibly the competition between the ions for absorption which would always result in a mutual reduction of intake. Now it is obvious that this reduction is not the same in all cases, otherwise the absorption when the different salts are present, as calculated in Table IV, would bear the same relationship to one another as do the ammonium absorption numbers of the salts, which is not so.

It has been shown by Ulehla (1930) that the permeability of a cell wall for ions depends inversely on their diameter; that is, directly on their mobility. Macallum (1929) has also suggested that the greater dispersion of potassium as compared with sodium in living matter can be related to its greater ionic mobility. It also seems to the author not unreasonable to suppose that the extent of mutual reduction of ion intake caused by ionic competition will depend, in part at least, on the relative ionic mobilities and coefficients of diffusion of the salts used.

It was pointed out by Mann (1924) that one would expect mutual hindrance in the passage of ions through a membrane to alter only the rate of equilibration and not the final position of equilibrium. However, it has been shown both by his experiments and ours (Table IV) that the reduction caused is more or less permanent. In this connection must be considered the recent work of Steward (1932) which suggests that the increase in the carbon-dioxide/oxygen ratio consequent upon the respiration of the tissue gradually results in a cessation of absorption. He has shown that a constant stream of air is necessary for the accumulation of bromide in potato tissue. That aeration results in a

greater intake of both ions of ammonium chloride has been mentioned previously by the author (1933). If the above suggestion is correct it will be evident that factors that cause a prolonged decrease of the rate of absorption of an ion by tissue in closed air systems will also decrease the amounts ultimately entering. There is then here no evidence that salt absorption does not take place through a membrane.

The second suggested factor can be explained in relation to Kahho's colloid precipitation theory. The kations used will increase or decrease the intake of ammonium by increasing or decreasing the permeability of the plasmatic membrane. According to Kahho (1921) this change in permeability results from monovalent salts causing a greater dispersion, and divalent or trivalent salts bringing about coagulation, of the colloids constituting the membrane.

It was thought possible at one time that antagonism could be explained on the grounds of adsorption of ions by the protoplasm. Thus if they are adsorbed it would be expected that on the introduction of another salt to a single salt solution not only would there be a mutual hindrance to intake but a permanent decrease owing to the appropriation, by the second salt, of some of the adsorptive surface. This decrease would be solely due to the operation of the competitive factor. The operation of the second factor could be explained as follows. The extent of intake of ions will depend on the area of the adsorbing surface, while the coagulation of the cell colloids by divalent and trivalent salts will decrease the surface area available for adsorption so that the amounts entering will be reduced. Monovalent salts, on the other hand, bring about a greater dispersion of the adsorbent with an increase in surface area and consequent intake of ions. Now although this explanation seemed quite feasible as applied to the experiments described in the earlier study it will be at once obvious that adsorption alone cannot adequately account for the results described in this paper. When tissue is immersed in a solution of a salt, adsorption of the kation would be expected to take place, and a corresponding area of adsorptive surface lost to the kation of the salt in which the tissue is subsequently immersed. Hence, in the experiments described above, subsequent immersion in ammonium chloride should give less intake of ammonium than when the two salts are present in the same solution, for under the latter conditions the ammonium ion is initially present to exert its competitive powers. In no experiment was this so; always the direct opposite was found.

It has been shown by Briggs (1932) that it is possible to account for the intake of salts by plant tissue on the basis of interchange of ions—kationic between the external solution and cytoplasm, and anionic between the external

solution and the cell sap. Attention has been drawn previously (Asprey, 1933) to the fact that lithium only causes an increase in the intake of ammonium from a mixed solution of these salts when it also causes an increase in the exosmosis of substances from the tissue. This fact may be related to the above possibility.

It is evident from the work of Ingold (1931) that the previous treatment of potato tissue with potassium, increases, and with calcium, decreases, the subsequent exosmosis of electrolytes by the tissue into conductivity water. From Ingold's data, taking the exosmosis in the control experiment, in which the tissue was previously treated with conductivity water, as 1, the mean value after treatment with calcium and potassium chlorides has been calculated and is given in Table V. The method used by Ingold for the previous treatment

Table V.—The exosmosis of electrolytes into conductivity water from potato tissue after previous treatment with solutions of potassium and calcium chlorides and conductivity water, that into the latter being taken as 1. (From Ingold's data.)

Time in hours.	Exosmosis in arbitrary units.		
	$CaCl_2$.	KCl.	Conductivity water.
2·5	0·78	1·42	1
5·5	0·75	1·46	1
8·5	0·80	1·53	1
Mean.	0·78	1·47	1

of the potato tissue was slightly different from ours, nevertheless a comparison of these values with the ammonium absorption numbers of the two salts shows that the salts in question have a remarkably similar effect both on subsequent exosmosis and subsequent absorption of ammonium by the tissue. This rather seems to indicate that when the passage out of ions from the tissue is increased, absorption would likewise be increased, and vice versa. To the author the relationship existing between these two phenomena seems significant and to provide further evidence that ionic interchange takes place, at least with kations. If this is so, such interchange will be an important factor in salt absorption and antagonistic action, and it may be that the value of the ammonium absorption number of a salt depends ultimately on whether the salt tends to increase or decrease the rate of exosmosis of electrolytes into the

external solution. If salt absorption does depend on ionic interchange then increase or decrease in the rate of salt absorption could be brought about by the alteration of the permeability of a protoplasmic membrane in the manner suggested by Kahho. There is here, however, no evidence as to whether the membrane should be regarded as the whole of the cytoplasm or a superficial layer of it.

The terms " antagonistic action " and " antagonism " are generally employed to express the effect that one substance has on the absorption of another when present in the same solution and they have tended to imply that this always results in a reduction of intake to a degree dependent on the nature of the substances used. It has been shown, however, that in some cases an increase in the intake of one of the participants may be brought about (Asprey, 1933). The work of Raber (1917) also seems to point to the fact that cases exist in which one salt may increase the toxicity of another. For this apparent reverse antagonism he has suggested the name " synergy." However, if the explanation of antagonistic action outlined above is accepted, there seems no reason further to complicate the terminology by introducing a new name for this apparently anomalous behaviour.

The writer wishes to acknowledge his indebtedness to Professor J. Walton for facilitating the work in many ways and also to Professor W. Stiles for his continued interest and helpful criticism.

Summary.

(1) The absorption of ammonium ions by potato tissue can be greatly altered by the previous immersion of the tissue in various salts. Sodium, potassium, and lithium chlorides increase, while calcium, magnesium, and aluminium chlorides decrease, the subsequent intake of ammonium as compared with that by tissue treated with distilled water. The increase caused by lithium is much greater than with either sodium or potassium chlorides.

(2) The relationship between the absorption of ammonium by treated and untreated tissue is approximately constant, at least up to 24 hours, for any one salt, and can be expressed by a factor that has been called, provisionally, the "ammonium absorption number" of that salt.

(3) Results support the suggestion that the antagonistic action of an ion depends on the balance existing between the specific effect of the ion on the absorptive capacity of the tissue and ionic competition for absorption.

(4) Evidence is brought forward that is not inconsistent with the idea that salt absorption by storage tissue is dependent on ionic interchange.

(5) So far as it provides an explanation of how the permeability of a plasmatic membrane may be altered, the colloid precipitation theory receives support from this work.

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Perforated Ray Cells.

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In certain woods, whose rays have extensive uniseriate margins, an individual marginal cell may sometimes be modified, by the perforation of its side walls, to connect two vessel segments on opposite sides of the ray. This feature does not appear to have been previously described though it is by no means uncommon; it has been observed by the authors in woods of many widely separated families. It is limited to woods with particular types of ray and vessel structure, but its occurrence seems to be primarily determined by the manner in which the vessels develop.

Large ray cells which appeared to have scalariform perforations in the side walls were first observed by the authors in the wood of *Lacistema aggregatum* (Berg.) Rusby. (Lacistemaceæ); examination of macerated material, fig. 1, has shown that the side walls of these cells are exactly similar to the end walls of the vessel segments, and though it has not been possible to demonstrate the absence of a membrane, the authors are convinced from a careful

study of the sections, that these walls are actually perforated, and that each forms a link between two vertical series of vessel segments. The course of the vessel is unusual; instead of continuing up and down between the rays, it periodically passes obliquely through a ray, and continues on the other side. The ray is usually uniseriate at the point at which it is crossed, and the ray cell involved becomes slightly swollen, rather in the manner of an oil or mucilage cell but without visible contents. The lateral walls are perforated, so that the cell resembles a very short vessel segment, and presumably functions as such. From the position and shape of these cells, however, it is clear that they have been derived from ray initials and not from fusiform initials. Attention was first drawn to this phenomenon by the presence of scalariform bars in the

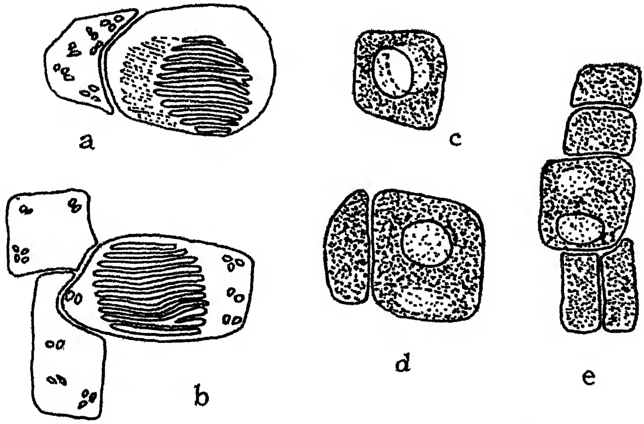


FIG. 1.—*Macerated material.* *a* and *b*, ray cells of *Lacistema aggregatum* Rusby, showing scalariform perforations; *c*, *d* and *e*, *Ptychopetalum anceps* Oliv., with simple perforations. (× 115.)

walls of cells which, from their shape, appeared at first sight to be oil or mucilage cells; simple perforations in such cells are obviously more easily overlooked, and it was not until a special search was made that they were found to be by no means uncommon, and to occur in woods of several different families.

In the following account of these cells, two woods in which they are numerous have been selected for description, *Lacistema aggregatum* (Berg.) Rusby, in which the perforations are scalariform, and *Ptychopetalum anceps* Oliv. (Olacaceæ), in which they are simple. The simple perforations in *Ptychopetalum* are shown in fig. 1, *c*, *d* and *e*; the cells are drawn from slides of macerated material, and the two perforations can be seen on the opposite walls of the cells.

Fig. 2 shows outline drawings of two pieces of tissue from macerated material. The position of a perforated ray cell relative to adjacent vessel segments in *Lacistema* is shown in A. A vessel segment *a* lies over some ray cells, the ray is passed through, and the vertical course of the vessel is continued by the segment *a'* on the other side of the ray; the ray cell *b* shows two scalariform perforations coinciding with those of the vessel segments *a* and *a'*. Fig. 2, B, shows a similar piece of tissue from *Ptychopetalum*; the vessel segment *a* lies

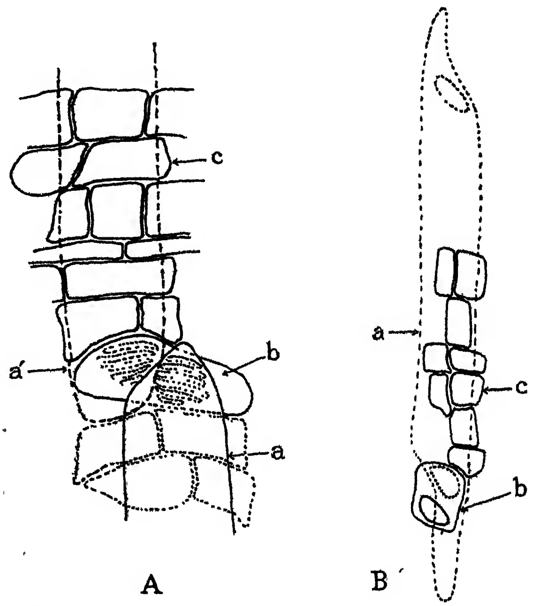


FIG. 2.—*Macerated material.* Groups of unseparated ray cells and vessel segments: A, *Lacistema aggregatum* Rusby; B, *Ptychopetalum anceps* Oliv. (*a*) vessel segment, (*b*) perforated ray cell, (*c*) ordinary ray cells. (× 75.)

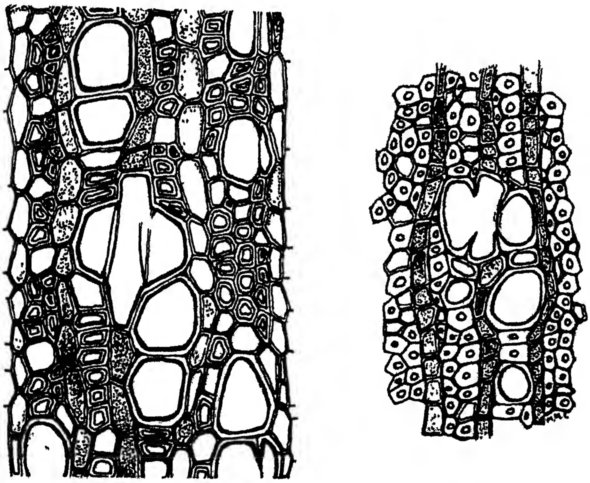
under some ray cells *c*, and at *b* there is a ray cell with two simple perforations; the perforation seen at the lower focus of the microscope (shown with a dotted line) coincides with that of the underlying vessel segment.

In macerated material in which the individual elements have not been completely separated, it is often possible to observe the perforated ray cell in position between the two vessel segments, but in longitudinal sections, one or other of the vessel segments is usually missing, and consequently the true nature of the phenomenon may easily be overlooked.

In cross-sections the perforations are not conspicuous but are easily distinguished if a search is made for them. Fig. 3 illustrates cross sections through the wood of *Lacistema* (A) and *Ptychopetalum* (B); the ends of two segments of

the same vessel can be seen separated by a perforated ray cell. In fig. 3, A, bars of the scalariform perforations and in B, the rims of the simple perforations are visible. In both drawings it is clear that the perforated ray cells are much larger radially than the normal ray cells, but are only slightly wider tangentially.

The change in the course of a vessel from one side of a ray to the other, and the linking up of the laterally displaced vessel segments by a cell of the ray, are most clearly seen in tangential section, and are illustrated in fig. 4, B. Fig. 4, A, shows a similar lateral displacement of the vessel segments in *Lacistema*, but only one vessel segment and one perforation into the ray cell



A B

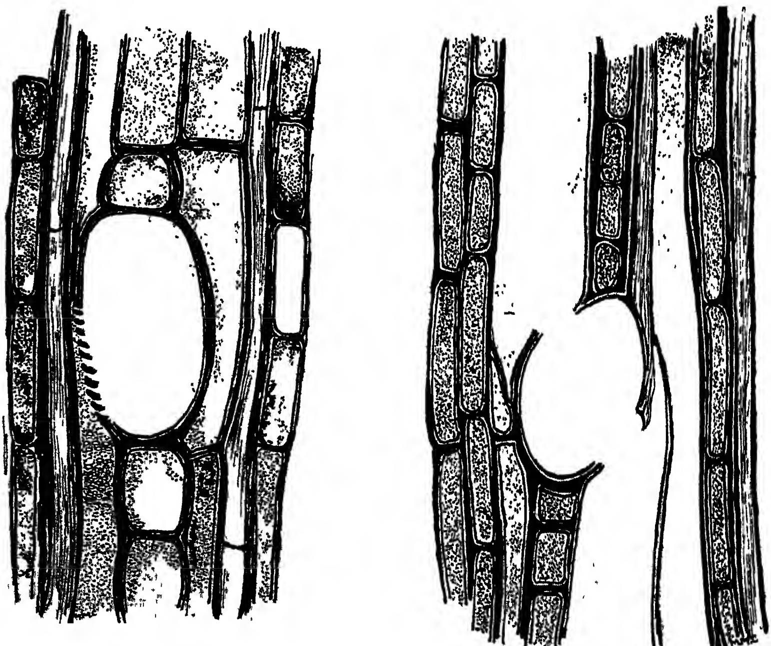
FIG. 3.—*Transverse section.* A, *Lacistema aggregatum* Rusby (× 130); B, *Ptychopetalum anceps* Oliv. (× 150).

can be seen; on the other side of the ray the section passes through a cell of the vasicentric wood parenchyma and not through the vessel itself.

The perforated ray cells are most conspicuous, and the details of the perforations most easily seen, on the radial section, but the tangential displacement of the vessel segments usually prevents the inclusion of both segments in the same radial section, and the continuation of the vessel on the opposite side of the ray is consequently seldom evident; it is, however, often possible to see both perforations in the ray cell, fig. 5D.

The perforations of the ray cells are scalariform where the end walls of the vessels have exclusively scalariform or mixed scalariform and simple perforations; where the vessel perforations are simple, those of the ray cells are

usually also simple, but may occasionally be scalariform. In both cases the scalariform perforations in the ray cells show a marked tendency to irregularity, of the type shown in fig. 6. This type of perforation is particularly common in association with vessels with simple perforations, for example, in the Rubiaceæ and some of the Euphorbiaceæ.



A B

FIG. 4.—*Tangential section.* A, *Lacistema aggregatum* Rusby; B, *Ptychopetalum anceps* Oliv. (× 300.)

Perforated ray cells have been found in the following woods :—

Apocynaceæ	*Funtumia africana* Stapf.†
	Gonioma kamassi E. Mey.
	**Odontadenia speciosa* Benth.†
	**Tabernæmontana citrifolia* L.†
	,, *grandiflora* Jacq.†
Araliaceæ	*Meryta sonchifolia* Linden and André.†
Calycanthaceæ..........	**Calycanthus floridus* L.†
Caprifoliaceæ	*Lonicera* sp.†

* Perforated ray cells extremely common.

† Correlated with herbarium material.

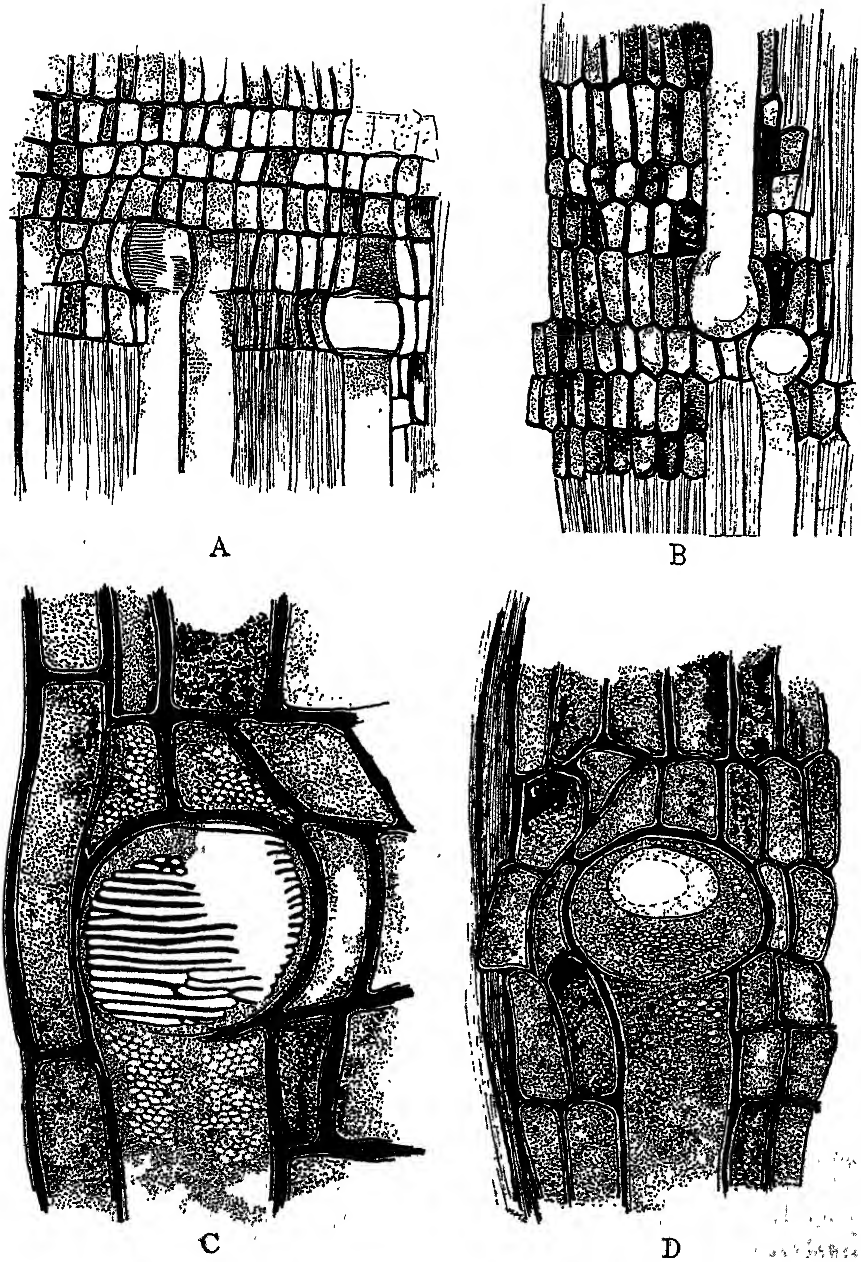


FIG. 5.—*Radial section.* A and C, *Lacistema aggregatum* Rusby; B and D, *Ptychopetalum anceps* Oliv. (A and B, × 150; C and D, × 300.)

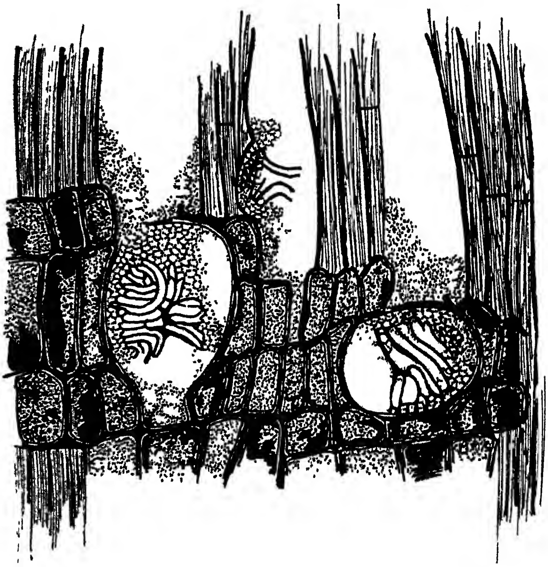


FIG. 6.—*Radial section*. Irregular perforations into ray cells, in *Antidesma* sp. (Euphorbiaceæ). (× 150.)

Celastraceæ	*Cassine capensis* L. var. *colpoon* D.C.
	,, *crocea* (Thunb) O. Kuntze.*
	Celastrus sp.
	†*Elæodendron australe* Vent.
	Euonymus europæus L.*
	Gymnosporia buxifolia Szysz.*
	Pleurostylia wightii Wight and Arn.
Euphorbiaceæ	*Antidesma* sp.
	Aporosa villosa Baill.*
	,, *villosula* Kurtz.*
	†*Blachia umbellata* Baill.
	†*Hemicyclia gardneri* Thwaites.
	Microdesmis puberula Hook.*
	†*Necepsia afzelii* Prain.*
Flacourtiaceæ	*Erythrospermum phytolaccoides* Gardn.
	Kiggelaria africana L.
	,, sp.
	†*Ophiobotrys zenkeri* Gilg.*
	Ryania speciosa Vahl.*
	Xylosma longifolium Clos.*

* Correlated with herbarium material.

Lacistemaceæ	**Lacistema aggregatum* (Berg.) Rusby.†
	* ,, *pedicellatum* Standl.†
	* ,, *nema* Macbr.†
	* ,, *rosediscum* Macbr.†
Malpighiaceæ	*Byrsonima crassifolia* H.B. and K.†
Olacaceæ	*Coula edulis* Baill.†
	Heisteria macrophylla Oerst.†
	Minquartia guianensis Aubl.†
	**Ptychopetalum anceps* Oliv.†
	**Strombosia javanica* Blume.
Passifloraceæ	*Androsiphonia adesnostegia* Stapf.†
Rubiaceæ	*Antirrhœa bourbonica* Gmel.
	Bertiera guianensis Aubl. †
	,, *racemosa* K. Schum.
	**Canthium ventosum* (L.) S. Moore.
	Calycophyllum candidissimum D.C.
	,, *spruceanum* (Benth.) K. Schum.
	**Corynanthe pachyceras* K. Schum.†
	Diplospora viridiflora D.C.
	Gardenia coronaria Buch.†
	,, *latifolia* Soland.
	,, *sessiliflora* Wall.†
	Guettardia foliacea Standl.†
	**Heinsia pulchella* K. Schum.
	Ixora parviflora Vahl.
	Luculia gratissima Sweet.†
	Mitragyna parviflora Korth.
	Pentagonia macrophylla Benth.
	**Posoqueria latifolia* Roem and Schult.†
	**Psychotria grandis* Sw.†
	Randia sp.†
	**Rondeletia buddleioides* Benth.†
	,, *cooperi* Standl.†
	Rudgea thyrsiflora Donn. Smith.†
	Sickingia sp.†

* Perforated ray cells extremely common.
† Correlated with herbarium material.

Rubiaceæ—(contd.)	**Vangueria griseum* Ridl.
	Vangueriopsis discolor Robyns.†
	Warscewicszia coccinea Klotzsch.†
Samydaceæ	*Casearia dinklagei* Gilg.†
	„ *præcox* Gris.
	* „ *sylvestris* Sw.†
	Homalium smythei Hutch. and Dalz.†
Saxifragaceæ	*Ribes glaciale* Wall.
Staphyleaceæ	**Turpinia pomifera* D.C.
Symplocaceæ	*Symplocos spicata* Roxb.†
Violaceæ	*Amphirrhox longifolia* Spreng.†

* Perforated ray cells extremely common,
† Correlated with herbarium material.

This list covers a considerable range of families, which are widely separated phylogenetically, and the woods themselves exhibit a corresponding diversity of structure. Even in a single species there may be great differences in the number of perforated ray cells in different specimens, which suggests that the occurrence of this phenomenon is due not so much to constant anatomical features characteristic of the species, but rather to more variable characters such as the orientation of the cambial initials and daughter cells.

Assuming the simultaneous formation of two vessel segments on opposite sides of a ray, it is comparatively easy to visualize the perforation of the ray cell to connect them, but it is extremely difficult to explain why the vessel segments should be so placed. Priestley (1932) has recently shown that in certain trees the differentiation of the vessels proceeds downwards, and it would appear, therefore, that the development of any undifferentiated cell into a vessel segment depends on the presence of a vessel segment above. If the end wall of the lowest differentiated vessel segment happens to be contiguous with a ray cell, it is possible that a perforation will be formed into the ray cell, and that this cell will, in turn, form a perforation on the opposite wall, and so lead to the differentiation of a further vessel segment on the opposite side of the ray.

Although the woods in which this phenomenon occurs are from such widely distributed families and exhibit such a range of structure, it is evident that they must have some common features which are associated with the occurrence of perforated ray cells; such features are to be found in the rays and vessels.

The rays are always high and have uniseriate margins of many cells, and it is amongst these marginal cells that the perforations usually occur ; in only two woods were perforated ray cells observed in multiseriate parts of the ray, fig. 7. In the woods in which the perforated ray cells are most numerous the rays range from 1 to 10 mm. high and are mostly higher than 3 mm. ; where the perforated ray cells are less numerous the rays are usually rather lower. The vessels are mostly small to very small, the tangential diameter of the largest vessels ranging from 50 μ to 100 μ. In a few woods the maximum diameter of the vessels was as high as 130 μ. The end walls of all the vessels are markedly oblique. The marginal cells of the rays are often extremely high, up to 240 μ, but it is noticeable that they are distinctly shorter in the woods where perforated ray cells are less numerous. It would appear, therefore, that the factor of importance in determining the frequency of perforated ray cells is not the actual dimensions of the marginal ray cells or of the vessels, but their relative proportions ; in all the woods it is possible to find ray cells which are higher than the tangential diameter of the largest vessels.

Fig. 7.—*Tangential section.* Vessel segments separated by two perforated ray cells in *Ophiobotrys zenkeri* Gilg. (Flacourtiaceæ). (× 250.)

These features are not confined to any particular systematic group, which may explain the occurrence of the phenomenon amongst such diverse families. The somewhat sporadic occurrence within the families is less easily explained. Though absence of perforated ray cells from many of the genera and species can be accounted for on anatomical grounds their absence from others cannot be so explained, and it would appear that there must be some other factor, which has so far not been observed, which determines the perforation of the ray cells.

It is difficult to show any phylogenetic connection between the families in which this phenomenon occurs, but it is suggestive that most of the woods (47 out of 62 genera) belong to the Celastrales and its offshoots, as suggested by Hutchinson (1926).

Acknowledgments.

Thanks are due to Dr. J. Burtt Davy, who checked the botanical names, and to the Department of Scientific and Industrial Research for a grant to Miss Chattaway.

Summary.

(1) An unusual type of ray cell was first observed in *Lacistema aggregatum*, the side walls being perforated and connecting two vertical series of vessel segments on opposite sides of a ray. Similar cells were subsequently observed in 74 species from 17 families.

(2) The anatomy of these woods is discussed in relation to the occurrence of this type of cell.

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Thrombase—Its Preparation and Properties.

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The preparation and properties of thrombase (fibrin ferment) were first described by Schmidt (1872). Serum was treated with 20 volumes of alcohol, and the mixture allowed to react for at least a fortnight and preferably for three months. The precipitate was dried over sulphuric acid and extracted with water. This aqueous extract, added to a liquid containing fibrinogen and paraglobulin, which did not coagulate spontaneously (*i.e.*, hydrocele fluid), rapidly produced coagulation. According to Schmidt the reaction was due to a ferment since the rapidity of coagulation was profoundly influenced by the amount of the solution added, although the total fibrin separated remained constant in amount.

Since that time a considerable amount of work has been done to investigate the phenomena observed in blood coagulation. As a result two distinct hypotheses have been advanced as to the place of thrombase in blood coagulation: (*a*) thrombase is the final cause of blood coagulation, and (*b*) thrombase is the result and not the cause of blood coagulation. The first hypothesis has been adopted by many observers notably Howell (1910), Bordet (1920) and Pickering (1928). The second hypothesis was originally put forward by Wooldridge (1886) who regarded blood coagulation as caused by the union of two antagonistic colloids obtained respectively from the blood and the tissues. This hypothesis has been adopted by Mills (1921), who considers that the action of thrombase only enters into the coagulation process in the concluding stages—that is, after a portion of the fibrinogen of the blood has reacted with a tissue protein to form fibrin. Nolf (1905) regards thrombase as an incomplete form of fibrin which is changed into the complete form by union with fibrinogen.

The chief experimental evidence in favour of the hypothesis of the secondary part played by thrombase in blood coagulation is the fact that Schmidt (1892) and many other observers have failed to produce intravascular coagulation when thrombase solutions are introduced into the blood stream of a living animal. This result appears to prove that thrombase is not a factor in the

normal coagulation of blood *in vivo* whatever effects it may produce *in vitro*. In this paper I shall show that the negative results of Schmidt and other observers were due to the feeble activity of their thrombase preparations and that the intravenous injection into a rabbit of 2 mg. of thrombase (prepared as subsequently described) quickly causes intravascular coagulation and death. I propose therefore to adopt the view that the formation and action of thrombase are essential factors in the coagulation of blood both in the living animal and outside of the body.

(1) *The Preparation of Thrombase from Mammalian Blood.*

The method I have adopted for the preparation of thrombase is based on three observations: (1) prothrombase is associated with fibrinogen in its precipitations, (2) prothrombase may be separated from precipitated fibrinogen by treatment with a dilute solution of calcium bicarbonate, and (3) prothrombase spontaneously changes into thrombase under certain conditions.

The method for the preparation of prothrombase from oxalated ox blood has been described in detail in a previous paper (Mellanby, 1930). In Section A (*d*) of this paper (p. 273) the precipitation of prothrombase from solution by the addition of $\overline{HA}$ 1 per cent. until the reaction is approximately p_H 5·3 is described. The prothrombase may be obtained as a compact mass by spinning in a high speed centrifuge. If this precipitate be dried at once, by means of acetone, a stable prothrombase preparation is obtained. If, however, the fluid be poured from the precipitate and the slightly moist prothrombase be left in the centrifuge tube for 24 hours it is converted spontaneously into a highly active thrombase. It may then be dried by means of acetone and preserved in an active condition for several months without deterioration. The average activity of thrombase prepared in this way is that 0·1 mg. coagulates 1 c.c. of oxalated plasma at 38° C. in 10 seconds.

This crude thrombase preparation may be purified by dissolving it in NaCl 0·5 per cent., keeping the reaction of the solution neutral by the cautious addition of alkali (the crude enzyme powder has a definite acidic reaction). The undissolved residue is spun off and the clear fluid acidified to p_H 5 by the addition of $\overline{HA}$ 1 per cent. An inactive precipitate is produced, the enzyme remaining in solution. This precipitate is removed by spinning and the thrombase is precipitated from solution by the addition of four volumes of acetone. The precipitate is spun off, washed twice with dry acetone and dried in a vacuum desiccator.

The following is the description of an experiment illustrating this latter procedure for the purification of thrombase.

200 mg. of spontaneously activated crude thrombase (acetone dried) were extracted with 20 c.c. NaCl 0·5 per cent. for 30 minutes. As solution proceeded the reaction was adjusted to p_H 7 by the cautious addition of Na_2CO_3. The undissolved residue was spun off and re-extracted with NaCl 0·5 per cent. until no more thrombase passed into solution. The active extracts were mixed and made up to 100 c.c. with NaCl 0·5 per cent. (0·02 c.c. of this thrombase extract coagulated 1 c.c. of oxalated ox plasma in 20 seconds), 1·5 c.c. of $\overline{HA}$ 1 per cent. was added to 100 c.c. of thrombase extract (the reaction became p_H 5). The precipitate produced was spun off. It contained only a trace of thrombase. The clear fluid was added to four volumes of acetone and the thrombase was precipitated. After 10 minutes the precipitate was spun off, washed twice with dry acetone, and dried in a vacuum desiccator. The weight of the final product was 120 mg. and the activity was such that 0·01 mg. coagulated 1 c.c. of oxalated plasma in 30 seconds.

(2) *Thrombase and Intravascular Coagulation.*

In 1886 Wooldridge described a series of experiments in which he produced either intravascular coagulation or negative phase blood after the intravenous injection of tissue extract into animals. A simple explanation of these apparently divergent results has been given in a previous paper (Mellanby, 1909). A rapid injection of tissue extract (thrombokinase) leads to a rapid formation of fibrin and hence intravascular coagulation results; a slow injection of tissue extract leads to a slow formation of fibrin the filaments of which do not coalesce but retract on to the walls of the blood vessels. In this latter condition the resultant blood does not clot when shed since it contains no fibrinogen.

From the observations recorded below it is clear that intravascular coagulation can be readily produced by the intravenous injection of thrombase and that the negative results of previous workers were due to the feeble activities of their thrombase preparations:—

(*a*) Rabbit 1925 grams. 3 mg. of thrombase dissolved in 5 c.c. Ringer solution were injected into the marginal vein of the ear. The animal died within 7 minutes, and showed intravascular coagulation of the portal system and vena cava the clot extending into the right auricle.

(*b*) Rabbit 2050 grams. 1·5 mg. of thrombase dissolved in 5 c.c. Ringer solution were injected into the marginal vein of the ear. Within 2 minutes the animal died. The vena cava and both ventricles were completely blocked with a dense clot.

A series of immunization experiments were carried out to determine whether permanently delayed blood coagulability could be produced by repeated injections of thrombase. The immediate effect of a small intravenous injection of thrombase is to augment the coagulability of the blood; this is followed within 20 minutes by a marked negative phase; then the coagulability of the blood returns to normal after a few hours. But even after the repeated injection of minimal quantities of thrombase over a period of several weeks the rapid injection of 2 or 3 mg. of thrombase invariably produced intravascular coagulation and death. No permanent change in the coagulability of the blood nor any immunity against thrombase could be produced by experimental immunization.

(3) *The Properties of Thrombase.*

The specific test for thrombase is its capacity to coagulate a solution of fibrinogen containing an excess of a soluble oxalate. In this paper plasma obtained from ox blood, to which potassium oxalate has been added to the extent of 0·2 per cent., is used as a test for thrombase.

There is no criterion of the purity of this preparation except the high uniform activity of a number of products obtained from different specimens of fresh oxalated ox plasma.

(*a*) *The Comparative Activities of Thrombase and various Snake Venoms.*—The venoms of certain viperine snakes possess the property of coagulating oxalated plasma—in fact these venoms contain thrombase in addition to their other toxic agents. Some of the venoms possess considerable coagulating activity and such venoms may produce death by intravascular coagulation. It was of interest therefore to compare this property of snake venoms with that of a thrombase preparation. Comparable solutions containing 0·5 mg., 0·05 mg. and 0·01 mg. of the venoms and thrombase were made up and added in varying amounts to 1 c.c. of oxalated plasma at 38° C.

The thrombase preparation was more than four times as active as the venom of the various varieties of *Lanceolatus*, and twice as active as that of the *Notechis Scutatus*.

Table I.

	Coagulation times for 1 c.c. of plasma.				
	Thrombase.	*L. atrox.*	*L. caseolutus.*	*L. alternatus.*	*Notechis scutatus.*
0·05 mg.	9 secs.	90 secs.	35 secs.	40 secs.	20 secs.
0·01 mg.	40 secs.	10 mins.	3·3 mins.	4 mins.	1·5 mins.

(*b*) *Solubility.*—Freshly prepared thrombase is a white amorphous powder freely soluble in water. Thrombase which has been kept in the dried state for some weeks may take an appreciable time to dissolve, although in the dry state it retains its activity for a considerable period of time without loss.

(*c*) *Composition.*—A solution of thrombase gives the following protein colour reactions—Biuret, Xanthoproteic and Millon. The glyoxylic reaction may be faintly positive. The product contains sulphur but no phosphorus. The absence of phosphorus is important in view of the assumed phosphatide nature of thrombokinase and the possible relation of this kinase to thrombase.

(*d*) *Destruction by Heat.*—Thrombase was dissolved in water, the solution was divided into three parts and adjusted to the reactions of p_H 6, 7 and 8. Portions of these solutions were heated to 40° C., 50° C. and 60° C. for 5 minutes, cooled, and their activities determined by observing the time taken for 0·1 c.c. of each solution (containing 0·025 mg. of thrombase) to coagulate 1 c.c. of oxalated plasma at 38° C. The figures in Table II were obtained.

Table II.

	Coagulation times, after heating the thrombase solutions to—		
	40° C.	50° C.	60° C.
p_H 6	20 secs.	25 secs.	No coagulation
p_H 7	20 ,,	22 ,,	,,
p_H 8	20 ,,	No coagulation	,,

Thrombase is destroyed after heating to 60° C. for 5 minutes whatever the reaction of the fluid in which it is contained. In an alkaline medium (p_H 8) this destruction is complete even at 50° C. It is evident that the degree of destruction of the thrombase is a function of both the temperature and the

duration of heating. The period of heating (5 minutes) was chosen since it allows a definite time for the effect of the increased temperature to manifest itself.

(*e*) *Dialysis.*—Thrombase slowly dialyses through a collodion membrane. This fact can be demonstrated by suspending a collodion tube containing a solution of thrombase in a vessel containing oxalated plasma, at a temperature of 38° C. After 2 hours a definite coagulum may be seen on the outer side of the membrane in contact with the plasma and the layer of fibrin progressively increases as the duration of dialysis is prolonged. Slow dialysis through a collodion membrane into distilled water may be demonstrated, but the quantity of thrombase demonstrable in the dialysate is never large, since the rate of destruction in a watery solution approximates to the rate of dialysis through the membrane.

(*f*) *Destruction by Acid and Alkali.*—Thrombase is readily destroyed by acid and alkali. The addition of acid to a thrombase solution in water results in its precipitation in an active form. If, however, an excess of acid be added to the solution to redissolve the precipitate then on bringing back the reaction to the neutral point the reprecipitated thrombase has entirely lost its coagulating action. These effects can be demonstrated by adding gradually 0·1 c.c. of 0·1 N HCl to 1 c.c. of a solution of thrombase in water.

The addition of NaOH to a thrombase solution does not precipitate it but after adding this alkali to the extent of 0·01 N the thrombase activity is not recovered on neutralization.

The destruction of thrombase by 0·01 N hydrochloric acid or sodium hydroxide illustrates the inherent fragility of the enzyme complex, since the solubility of the inactivated thrombase when reprecipitated is not different from that of the original thrombase preparation.

(*g*) *Precipitation by Acetone, Alcohol and Ammonium Sulphate.*—The precipitation of thrombase from a solution in NaCl 0·5 per cent. by acetone has been described in the method of preparation. In this precipitation it is advisable to add the enzyme solution to the acetone to ensure rapid flocculation. It is difficult to precipitate thrombase dissolved in water by means of acetone, in fact the volume of acetone has to be so large that the formation of a precipitate is difficult to observe.

Similar facts hold good for alcohol precipitation. Large volumes of absolute alcohol are unable to precipitate from solution thrombase dissolved in water. But thrombase dissolved in NaCl 0·5 per cent. is readily precipitated when added to four volumes of dried absolute alcohol.

Thrombase is precipitated from solution by full saturation with ammonium sulphate. The precipitated thrombase is found to have preserved its activity when redissolved. Thrombase, dissolved in water, is also precipitated by the salts of heavy metals as copper sulphate. The precipitate when redissolved in dilute Na_2CO_3 is found to have preserved its original activity.

(4) *The Action of Thrombase on Plasma.*

A series of experiments were carried out to determine the relation of the time of coagulation of plasma by thrombase to (*a*) the quantity of thrombase added, (*b*) the degree of dilution of the plasma, and (*c*) the temperature of the reacting fluids.

(*a*) *The Relation of the Time of Coagulation to the Quantity of Thrombase.*—Generally speaking the time of coagulation is inversely proportional to the amount of thrombase added—the same proportionality being observed as in the coagulation of milk by gastric or pancreatic rennins.

Varying quantities of thrombase were added to 1 c.c. of oxalated plasma the volume being kept constant by the addition of NaCl 0·5 per cent. The coagulation times given in Table III at 38° C. were observed.

Table III.

Plasma.	NaCl 0·5 per cent.	Thrombase.	Coagulation time.
c.c.	c.c.	c.c.	secs.
1	0·95	0·05	135
1	0·9	0·1	60
1	0·8	0·2	26
1	0·6	0·4	15
1	0·2	0·8	8
1	0·0	1·0	6

These figures show that the times of coagulation are inversely proportional to the amounts of enzyme added except in the first and last experiments. When the time of coagulation extends beyond 3 minutes the inverse relation is lost, the time of coagulation being prolonged out of proportion to the quantity of enzyme added. Similarly it is difficult to get a less coagulation time than 5 seconds however much the quantity of enzyme added probably owing to the time taken for the thrombase to associate itself with the fibrinogen.

(*b*) *The Influence of the Dilution of Plasma on the Time of Coagulation by Thrombase.*—The dilution of a plasma thrombase mixture by NaCl 0·5 per

cent. prolongs the time of coagulation to a degree proportionate to the dilution. This statement does not hold true for high dilutions of plasma.

Three series of experiments were carried out in which the final dilutions of the plasma thrombase mixtures were 4 c.c., 6 c.c., and 8 c.c. respectively, these volumes being made by the addition of the necessary quantities of NaCl 0·5 per cent. In every experiment 1 c.c. of plasma was present and the thrombase was varied from 0·1 c.c. to 0·5 c.c. The strength of the thrombase solution was such that 0·1 c.c. contained 0·01 mg. thrombase. The coagulation times, Table IV, at 38° C. were observed.

Table IV.

Plasma.	Thrombase.	0·5 per cent. NaCl.	Coagulation times for volumes of—		
			4 c.c.	6 c.c.	8 c.c.
c.c.	c.c.		secs.	secs.	secs.
1	0·1	Up to required volume	150	255 (*a*)	430 (*b*)
1	0·2		75	95	220 (*c*)
1	0·3		48	70	92
1	0·4		35	47	75
1	0·5		27	40	54

In each series of experiments the law of the inverse proportionality between the times of coagulation and the amount of thrombase added may be seen to hold except for the high dilutions of thrombase (*a*, *b*, *c*). A comparison shows that the times of coagulation are proportionate to the degrees of dilution. Thus suppose we take the average of the product of the time of coagulation and the quantity of enzyme in each experiment (excluding *a*, *b*, *c* in which the thrombase is so dilute that the inverse relation between the quantity of thrombase and the time of coagulation is lost) we get the results given in Table V.

Now 144 : 197 : 282 :: 4 : 5·5 : 7·8, or, as an average approximation, the times of coagulation are directly proportional to the degrees of dilution of the plasma thrombase mixtures.

(*c*) *The Temperature Coefficient.*—A fall of temperature diminishes the rate of coagulation of plasma by thrombase, but even at 0° C. coagulation may take place rapidly in the presence of a large amount of enzyme. Above 50° C. the rate of coagulation is diminished owing to the destruction of the enzyme. The figures in Table VI show the rates of coagulation of 1 c.c. of oxalated plasma at varying temperatures by 0·05 mg. and 0·025 mg. of thrombase.

Table V.

Thrombase.	Product of coagulation time and quantity of thrombase for volumes of—		
	4 c.c.	6 c.c.	8 c.c.
c.c.			
0·1	150	— (*a*)	— (*b*)
0·2	150	190	— (*c*)
0·3	144	210	276
0·4	140	188	300
0·5	135	200	270
Average	144	197	282

Table VI.

Temperature.	Coagulation times.	
	0·05 mg. thrombase.	0·025 mg. thrombase.
° C.	secs.	secs.
0	78	120
10	33	60
20	27	50
30	15	26
40	10	22
50	16	35

The comparatively small increase in the coagulation time between 30° C. and 10° C. is remarkable. It is due to the fact that two distinct processes are involved in coagulation: (1) the action of the enzyme on the fibrinogen converting it into fibrin, and (2) the aggregation of the fibrin particles into an insoluble coagulum. Probably the rate of enzyme action is inhibited to the usual degree by the fall in temperature whilst the solubility of the fibrin in the salt solution is diminished as is the case with all gobulins. These two temperature processes tend to balance one another—hence the comparatively small effect of temperature on the entire process.

The figures in Table VII giving the times of coagulation of plasma by varying quantities of thrombase at 0° C. show that at this temperature the law of inverse proportionality holds true.

Table VII.

Plasma.	Thrombase.	NaCl 0·5 per cent.	Coagulation time.
c.c.	c.c.		secs.
1	0·1	0·9	132
1	0·2	0·8	90
1	0·3	0·7	70
1	0·4	0·6	55
1	0·5	0·5	40
1	0·6	0·4	35

(5) *The Adsorption of Thrombase when Fibrinogen is Converted into Fibrin.*

Whatever quantity of thrombase is added to oxalated plasma practically the whole of it disappears from the expressed fluid within a few minutes after the conversion of the fibrinogen into fibrin.

Thus 1 c.c. of plasma was coagulated by 0·1 mg. of thrombase in 8 seconds. After coagulation the fibrin was removed and the thrombase activity of the expressed fluid (0·2 c.c.) was tested on plasma (1 c.c.) after varying times. After 1 minute coagulation by the expressed fluid took place in 25 seconds; after an interval of 3 minutes no coagulation occurred—the excess of thrombase had entirely disappeared from the expressed fluid. This phenomenon has been discussed in detail in a previous paper and the normal presence of a large quantity of antithrombase in plasma has been assumed to account for the observed facts. In order therefore to determine the fate of thrombase when fibrinogen is converted into fibrin it is necessary to add the thrombase to the fibrinogen dissolved in sodium chloride. Under these circumstances there is no antithrombase present in the fibrinogen solution and any excess of thrombase left after coagulation has taken place remains intact.

Fibrinogen was precipitated from oxalated plasma by dilution with 10 volumes of distilled water and the addition of $\overline{HA}$ 1 per cent. until the reaction was p_H 5·5. The precipitate was spun off, dissolved in a volume of NaCl 0·8 per cent. equal to that of the original plasma and used as the solution of fibrinogen in the following experiment, Table VIII.

(A) To 5 c.c. of the fibrinogen solution varying quantities of thrombase were added, the total volumes being kept constant by the addition of NaCl 0·8 per cent.

In every case the amount of thrombase added was so large that coagulation took place practically at once.

Table VIII.

Fibrinogen.	NaCl 0·8 per cent.	Thrombase.	Coagulation time.
	c.c.	c.c.	secs.
(*a*) 5 c.c.	3	1	3
(*b*) 5 c.c.	2	2	2
(*c*) 5 c.c.	1	3	2
(*d*) 5 c.c.	0	4	2

(B) The fluids were expressed from the fibrin in the above mixtures and the quantities of thrombase in these residual fluids determined.

Table IX.

Plasma.	Residual fluid.	Coagulation time.
c.c.	c.c.	secs.
1	0·2 (*a*)	25
1	0·2 (*b*)	13
1	0·1 (*c*)	17
1	0·1 (*d*)	13

As a first approximation and assuming the accuracy of the inverse law of the time of coagulation to the quantity of enzyme present it is evident that the relative quantities of the enzyme contained in the expressed fluids *a*, *b*, *c* and *d* is

$$a : b : c : d :: 1 : 2 : 3 : 4.$$

Therefore the expressed fluids have the same ratio of thrombase content as was added to the original fibrinogen solutions.

Further the figures allow a simple means of calculating the amount of thrombase removed in each case when fibrin is formed from fibrinogen. On the assumption that 1 c.c. of the original thrombase solution contained 100 units of enzyme these calculations give the results shown in Table X.

Table X.

Fibrinogen.	Thrombase added.	Thrombase left in expressed fluid.	Thrombase removed.
	units.		
(*a*) 5 c.c.	100	60	40
(*b*) 5 c.c.	200	120	80
(*c*) 5 c.c.	300	180	120
(*d*) 5 c.c.	400	240	160

It is evident that the thrombase lost is removed with the fibrin since the fibrin from (*d*), dried and extracted with slightly acidified water (p_H 6), after 24 hours gave a solution which readily coagulated oxalate plasma. These figures show that the quantity of enzyme adsorbed by the fibrin is determined by the original concentration of thrombase added to the fibrinogen solution and the preparation of thrombase by Gamgee's (1879) method depends partly upon the resolution of this adsorbed thrombase from the fibrin by means of sodium chloride.

Discussion of Results.

The hypothesis of Wooldridge that the coagulation of blood depends upon the union of two fibrinogens A and B to form fibrin has indicated that coagulation is caused by the mutual precipitation of two antagonistic colloids. Two statements have been put forward by Rettger (1909) in support of this hypothesis: (*a*) the heat stability of thrombase, and (*b*) the direct relation between the weight of thrombase added to a solution of fibrinogen and the quantity of fibrin formed.

(*a*) In regard to the heat stability of thrombase no evidence has been obtained that the thrombase described in this paper is otherwise than heat labile—in fact thrombase in a slightly alkaline medium is destroyed after heating to 50° C. for 5 minutes.

(*b*) No evidence has been obtained that the quantity of fibrin formed is related to the amount of thrombase present when the thrombase exceeds a certain minimal concentration in which the time relations of coagulation obey the inverse law. In every case the whole of the fibrinogen is converted into fibrin. Since 0·01 mg. of thrombase converts the whole of the fibrinogen in 1 c.c. of plasma (about 5 mg.) into fibrin in 30 seconds it is clear that even if all the thrombase were adsorbed by the fibrin the weight formed would not be appreciably affected by the weight of thrombase—or the variation in weight of fibrin with varying weights of thrombase would be within the limits of experimental error. But when the amounts of thrombase added are so small that the law of inverse proportionality no longer holds then the weight of fibrin appears to be determined directly by the quantity of thrombase added. Under these circumstances the first formed fibrin adsorbs the greater part of the enzyme present and the resultant slow action of the residue accompanied by its slow destruction determines the practical cessation of coagulation.

There are certain definite facts against the hypothesis that thrombase and fibrinogen are two antagonistic colloids which by their union form fibrin. In the first place the isoelectric points of fibrinogen and thrombase are practically

identical. Both of them are precipitated at the same reaction, *i.e.*, p_H 6. They are unable therefore to react as two antagonistic colloids. Further there is no direct experimental evidence that two oppositely charged colloids precipitate one another according to a simple law of direct proportionality such as described for thrombase and fibrinogen (*cf.* Mellanby and Anwyl-Davies, 1923).

The quantitative facts observed in the coagulation of fibrinogen by thrombase are parallel to those described for the coagulation of caseinogen by gastric rennin or pancreatic rennin. There is one essential difference however in the two processes—calcium ions are necessary for the precipitation of paracasein as casein, whereas the formation of a fibrin gel occurs readily not only in the absence of Ca ions but in the presence of a considerable quantity of a soluble oxalate. This parallelism of the coagulating phenomena observed in milk and blood is shown in the identity of the following approximate laws in both cases: (*a*) the inverse proportionality between the times of coagulation and the qualities of enzyme added, (*b*) the direct relation of the time of coagulation to the degree of dilution of the fluid, (*c*) the removal of the enzyme from the solution in proportion to its original concentration, and (*d*) the recovery of the enzyme from the coagulum by various solvents. Further the high potency of rennin as a coagulating agent has its counterpart in the activity of thrombase. As may be seen in the preceding experimental results a concentration of 1 : 100,000 of thrombase in plasma produces coagulation in 30 seconds.

The hypothesis which I put forward in 1908 on the action of thrombase appears to accord with the additional facts described in this paper—thrombase is a proteoclastic enzyme which acts on fibrinogen splitting it into two parts, an insoluble protein, fibrin, which forms the coagulum and a soluble protein, serum globulin, which remains in solution.

Summary.

(1) Thrombase may be prepared from oxalated ox blood by (*a*) the spontaneous activation of prothrombase prepared in the way previously described, (*b*) the solution of this thrombase in NaCl 0·5 per cent., and (*c*) precipitation of the thrombase by four volumes of acetone.

(2) 1 mg. of thrombase coagulates 100 c.c. of oxalate plasma in 30 seconds.

(3) The intravenous injection of 2 mg. into a rabbit causes intravascular coagulation and death.

(4) Thrombase is a protein, soluble in water. The enzyme, dissolved in water, slowly dialyses through a collodion membrane. It is destroyed by HCl 0·01 N and NaOH 0·01 N.

(5) The action of thrombase on plasma shows the following relations: (*a*) the time of coagulation is inversely proportional to the quantity of enzyme, and (*b*) the dilution of a plasma thrombase solution prolongs the time of coagulation to a degree proportionate to the dilution.

(6) When fibrinogen is converted into fibrin by thrombase a quantity of thrombase is removed from solution in proportion to its original concentration. Gamgee's method for the preparation of thrombase from fibrin depends partly upon the resolution of this adsorbed thrombase by NaCl 8 per cent.

(7) The hypothesis is put forward that thrombase is a proteoclastic enzyme which splits fibrinogen into fibrin and serum globulin.

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595 . 315 Dodecolopoda.

A Dodecapodous Pycnogonid.

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Among the Pycnogonida obtained by Sir Douglas Mawson, F.R.S., on his latest voyage to the Antarctic is a specimen presenting the novel and remarkable feature of having six pairs of legs. It is of relatively gigantic size, spanning 20 inches from tip to tip of its outstretched legs, and represents a new species which we propose to refer to a new genus. The purpose of this paper is to give a preliminary diagnosis of the genus, a brief description of the specimen for systematic purposes, and a discussion of its bearing on the morphology and phylogeny of the Pycnogonida.

Dodecolopoda, gen. nov.

Differing from *Decolopoda* only in possessing six instead of five trunk-somites, each bearing a pair of legs.

Genotype—Dodecolopoda mawsoni, sp. n.—The affinities of this genus are further discussed below.

Dodecolopoda mawsoni, sp. n.

Occurrence.—Stn. 107. Lat. 66° 45′ S., long. 62° 03′ E.; on a submarine bank off the coast of MacRobertson Land, depth 219 m.; February 16, 1931. One male (Holotype).

Description.—*Trunk* unsegmented but with inconspicuous inter-segmental grooves on dorsal surface; greatest width across second or third lateral processes equal to its length. Lateral processes in contact distally, the three anterior pairs slightly longer than the three posterior (fig. 1, A).

Cephalon broader than long, occupying the whole space between the first pair of lateral processes. Ocular tubercle much less than half width of cephalon, bluntly conical, wider than high. Eyes four, subequal.

Proboscis three-fourths as long again as trunk, decurved distally; subcylindrical for the first third, then expanding to twice the width and narrowing a little to the tip (fig. 1, B).

Abdomen slender, subcylindrical, straight, reaching a little beyond middle of second coxa of last legs.

Chelophore rather shorter than proboscis; first segment of scape long, slightly expanded distally; second segment one-third as long as first; chela twice as long as second segment, with short palm and strongly arched fingers which cross at the points (fig. 1, B.)

Palp of nine segments, exceeding the proboscis by the four terminal segments; second segment as long as fourth and fifth together; fourth segment, containing the palpal gland, slighly inflated (fig. 1, A and B).

Oviger of ten segments with minute terminal claw; fourth and sixth segments subequal, about twice as long as fifth; four terminal segments subequal, armed with numerous special spines set in six to nine rows.

Legs twelve, long and stout; first pair rather shorter than the others which are subequal. Femur considerably shorter than the subequal tibiæ; tarsus about twice as long as the propodus, claw not quite half as long (fig. 1, C).

On the ventral surface of each second coxa, in its distal third, is a minute genital pore, hardly to be distinguished from the sockets of the minute setæ around it. The size and position of the pore indicate that the specimen is a male.

Setæ are minute and scattered except on the longer segments of the legs where they are conspicuous, mostly not longer than half the diameter of the segment which bears them, and arranged in six longitudinal rows (fig. 1, C).

Injuries, etc. The left chelophore has been cut off near the base and the stump healed over. The fifth right leg has the terminal segments shorter than in the other legs, as if regenerated. There are small patches of encrusting Polyzoa on the body and legs and the under surface of the body bears a mass of rounded grains, apparently the spawn of some animal.

Measurements, in millimetres :—

Length of proboscis	31·5
Greatest width, proboscis	6·5
Length of trunk	18·0
Width across third lateral processes	18·0
Length of abdomen	10·5

Comparison with other Species.—Apart from its possession of an additional leg-bearing somite, and its much greater size, the new species differs from both the species of *Decolopoda* in having (1) the proboscis much longer relatively to the trunk; (2) the tarsus much longer and the claw much shorter relatively

to the propodus; and (3) the legs distinctly setose. It resembles *Decolopoda australis* in having the ocular tubercle much less than half the width of the

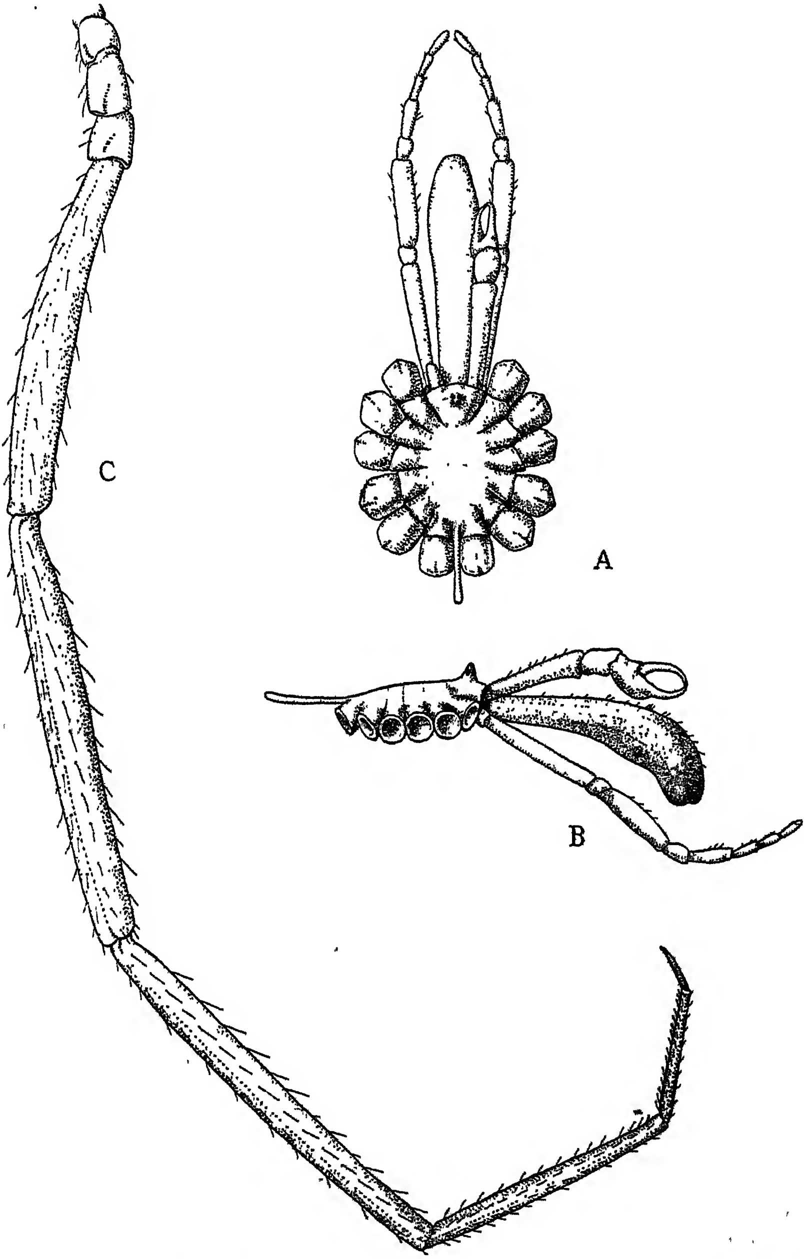

FIG. 1.—*Dodecolopoda mawsoni g. et.* sp. n. (natural size). A, Dorsal view of body with palps, right chelophore and first coxæ. B, Lateral view of body with chelophore and palp, oviger omitted. C, Fourth leg of left side.

Chelophore.		Palp.	Leg of fourth pair.	
First segment	13·5	Lengths of segments in order from the second—	Total length	240·3
Second segment	4·6	15·0	Coxæ (together)	16·0
Chela	10·0	2·3	Femur	48·5
		11·5	First tibia	57·5
		3·5	Second tibia	58·2
		5·8	Tarsus	36·1
		3·3	Propodus	17·0
		3·3	Claw	7·0
		3·5		

cephalon and the chela with short palm and strongly arched fingers. In the long and slender first segment of the chelophoral scape it approaches *D. antarctica.*

Secondary Metameric Instability in Pycnogonida.

The discovery of a Pycnogonid with six leg-bearing somites does not really involve any important modification of the problem presented by the ten-legged species. It does, however, lay emphasis on certain aspects of that problem.

It may be pointed out, in passing, that there is no reason for supposing *Dodecolopoda* to be merely an individual abnormality. That suggestion was, indeed, made more than once with regard to the decapodous species. Loman (1905, p. 723) called Eights's *Decolopoda* "ein irrationelles Monstrum," and Bouvier (1906, *a*, p. 17) at first regarded the possession of five pairs of legs by his "*Colossendeis*" *antarctica* as "une anomalie sans grande valeur." All the decapodous species, however, are now represented by several, and some by many specimens, and there is no reason to suppose that the holotype of our new species will remain unique. Further, as already pointed out, even if we disregarded its additional somite and appendages, *Dodecolopoda mawsoni* would still be distinguishable from both the known species of *Decolopoda.* On the other hand, the possibility of an additional pair of appendages appearing as an isolated variation or abnormality is shown by Dogiel's (1911) description of a very young specimen of *Chætonymphon spinosum* possessing a rudimentary pair of appendages behind the fourth pair of legs.

One of us (Calman, 1909, p. 691; 1915, p. 7) has already argued in favour of the view (first suggested by Dr. G. H. Carpenter, 1905) that the decapodous genera of Pycnogonida present a secondary modification of the octopodous type, in opposition to the opinion of Bouvier and others that these genera are primitive survivals. The facts that these three genera are widely separated

in the classification, that each is paired with a very similar but octopodous genus, and that *Pentapycnon* with its "normal" neighbour *Pycnogonum* are among the most specialized members of the group, still seem to us conclusive in this respect.

Dodecolopoda, with its very close resemblance to *Decolopoda*, now brings the interesting suggestion that, once the octopodous standard was departed from, a state of metameric instability set in and the Pycnogonida became, to use Lankester's term, "anomomeristic." Before the discovery of the decapodous species the metameric pattern of the Pycnogonida seemed to be as firmly established as that of Insecta or Arachnida, remaining constant throughout a considerable range of differences in detailed structure.* When this constancy had been disturbed, however, the appearance of six instead of five somites might represent a comparatively minor variation.

Attention has been called (Calman, 1915, p. 9) to the parallel presented by the six-gilled Elasmobranch *Pliotrema* and its five-gilled relative *Pristiophorus* to *Pentapycon* and *Pycnogonum* respectively. It is tempting to compare, similarly, *Decolopoda* and *Dodecolopoda* with the Notidanid sharks *Hexanchus* and *Heptanchus*, although we are assured that in these the larger number of gill-arches is primitive and not secondary as it appears to be in *Pliotrema*. A closer parallel is afforded, as Dr. Tate Regan has pointed out to us, by the multiplication of metameres in Teleostean fishes. In these, the presence of 24 vertebræ appears to be a primitive character in several lines of descent. This number may be constant within a family (*e.g.*, Sparidæ) or within a genus or a group of genera (*e.g.*, within the family Serranidæ) and shows no variation between individuals. When the number is increased, however, no further position of stability is reached; the number of vertebræ shows diversity within the family or the genus, and variation within the species.

It may not be without significance that in *Decolopoda antarctica*, alone among the Pycnogonida, the number of segments in the palp is variable (Calman, 1920, p. 243; Gordon, 1932, pp. 11 and 130). This suggests that the metameric instability which we believe to have affected the trunk-somites may possibly have influenced also the segmentation of these appendages.

Affinities of the Pycnogonida.

The metameric pattern of the (octopodous) Pycnogonida differs from that of the Arachnida by the presence of an additional pair of appendages between

* About 40 to 45 genera and 400 species of octopodous Pycnogonida are now known.

the chelophores and the first legs. At the same time their numerous resemblances to the Arachnida—the chelate preoral appendages, the four pairs of walking-legs, the intestinal diverticula, the structure of the eyes, and many other details—point to an affinity with the more specialized Arachnida, not with a primitive stock in which the metameric pattern had not yet been defined. One of us (Calman, 1929, p. 103) has hazarded the suggestion that full value might be given to the Arachnid resemblances of the Pycnogonida if we were at liberty to assume a disturbance of the metameric pattern affecting the number of the cephalic metameres, similar to the disturbance which we believe to have affected the trunk segments in the decapodous and dodecapodous genera.

The phylogeny of the Arthropoda, as far as it can be inferred from the classification of existing forms or from palæontology, shows that such disturbances of an established metameric pattern must have been infrequent. We believe, however, that they have occasionally happened, and that it is not illegitimate to invoke their aid when attempting to solve some of the more difficult problems of arthropodan phylogeny.

Until 1929 palæontology had no contribution to make to the history of the Pycnogonida. In that year, however, Professor F. Broili (1929, also 1930) described, from the Lower Devonian roofing slates of the Hunsrück district in Rhenish Prussia, a fossil which he named *Palæopantopus,* and believed to be a representative of the Pycnogonida. We have had an opportunity of studying this remarkable form, not only in some excellent photographs of the type which Professor Broili has been kind enough to send to us, but also in a very perfect cast of the original which he has presented to the Geological Department of this Museum.

Palæopantopus has certainly a great resemblance to a Pycnogonid in general form, with its very small body and four pairs of long legs, as well as in the disposition of the main segments composing the latter. At the same time the following considerations make us hesitate to accept its reference to the Pycnogonida as final.

1. The absence of any unmistakable trace of the proboscis which is so conspicuous in all existing members of the group, as well as of chelophores and palps.

2. The series of parallel transverse grooves on the lateral processes of the body (or possibly the basal segments of the legs). We know of nothing even remotely resembling these in any living species.

3. While the three long segments of the leg would correspond to the femur and first and second tibiæ of the Pycnogonid, the three short coxal segments of

the legs are very obscure, and, what is perhaps the most important character of all, the terminal part of the leg is almost certainly divided into not less than five short articulations.

While the affinities of *Palæopantopus* must therefore be regarded as undecided, it should be pointed out that if it be really a Pycnogonid it carries back the octopodous type to the remote period of the Lower Devonian.

Palæoisopus, from the same formation, described by Broili (1928, p. 201) as an Isopod, was later referred by him to the Pycnogonida (Broili, 1932). We have examined casts of this remarkable form also and find no reason for supposing it to be related to either the Pycnogonida or the Isopoda. It appears to show no clear indications of affinity with any other Arthropod known to us. If it is a Crustacean it must be a very specialized offshoot from some primitive and extinct group.

Classification.

Since our new species would undoubtedly be referable to the genus *Decolopoda* if it were not for its possession of an additional leg-bearing somite, and since we believe that the difference between five and six somites is of less phylogenetic importance than that between four and five, it would have been quite logical to include the species in *Decolopoda.* We have established a new genus for it mainly on grounds of practical convenience and because taxonomic categories can never be more than an approximate summary of phylogenetic conclusions.

While *Pentanymphon* and *Pentapycnon* have been included with the related octopodous genera in the families Nymphonidæ and Pycnogonidæ respectively, *Decolopoda* has been made the type of a family Decolopodidæ (Cole, 1905, p. 409; Bouvier, 1906*b*, p. 20) separated from the Colossendeidæ only by having chelophores present in the adult. With this we are unable to agree. Chelophores like those of *Decolopoda*, with a two-jointed scape, are present in the larval stages of *Colossendeis* and may be retained in specimens that have reached a considerable size. In at least one instance they were present in a specimen that was mature, or nearly so, since the genital openings were distinct, although other specimens presumably of the same species were without chelophores (*Colossendeis gracilis*, Hoek, 1881, pp. 69-70). We do not consider that the persistence of chelophores can be regarded as a family character. In the family Ammotheidæ, where the chelophores are usually imperfect or absent in the adults, two species of the genus *Ammothea* have been found to have well-developed chelophores in specimens that appeared to be sexually mature (Bouvier, 1913, p. 125; Gordon, 1932, pp. 95 and 97).

We propose, therefore, to include *Decolopoda* and *Dodecolopoda* in the family Colossendeidæ, amending the diagnosis of the family (from which we omit *Pipetta*) to read as follows :—

Cephalon short, neck absent; proboscis large, usually exceeding trunk length. Trunk of 4-6 somites, fused or free. Chelophores with two-jointed scape in larva, sometimes persisting in adult. Palp with 8-10, usually 9 segments, inserted on a ventral process similar to and usually contiguous with that which bears the oviger. Oviger large in both sexes, with 10 segments and a terminal claw which is often much reduced ; several rows of special spines on segments 7-10. Legs 8-12 ; coxal segments relatively short, a genital pore on each second coxa in both sexes ; auxiliary claws absent. Species often of large size.

Summary.

(1) *Dodecolopoda mawsoni*, a new species representing a new genus of Pycnogonida, is described. It was obtained by Sir Douglas Mawson in the Antarctic, and has the remarkable peculiarity of possessing six leg-bearing somites.

(2) It is suggested that the ten-legged and twelve-legged Pycnogonida originated by the development of instability in the metameric pattern of octopodous forms ; also that the Pycnogonida as a whole may owe their origin to an analogous disturbance of the cephalic metamerism of normal Arachnida.

(3) Broili's *Palæopantopus* from the Lower Devonian is discussed and reasons are given for suspending judgment as to its Pycnogonid affinities. *Palæoisopus* from the same formation shows no indication of affinities with the Pycnogonida.

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612 . 76 : 597

Directional Control of Fish Movement.

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[Plates 3–7.]

One of the most striking features of a fish's movement is the rapidity with which the animal can change the direction of its motion; in most cases the process is carried out much too rapidly to permit accurate visual observations. When the turning movements are effected slowly, it is not infrequently seen that the pectoral fins represent an important and active part of the mechanism, but in very few cases—if indeed any—can they be regarded as the primary controls of rapid directional changes. In other cases, reasonably rapid changes are seen to be effected by a sudden deflection of the caudal fin towards the side to which the fish turns. The observation of a large variety of fish leaves no doubt that it is flexures of this type which are responsible for the most rapid and most effective changes in the direction of fish motion. When a Goldfish or a Rudd turns slowly, the tail appears to strike towards the side to which the

animal turns, and appears to move rapidly through the water whilst the head of the fish remains relatively undisplaced during the process. This impression is erroneous, and is due to the fact that the eye observes movements of the tail relative to the head instead of observing its motion relative to the background of the fish. It is, of course, only movements of the fish relative to the background which are of significance during changes in direction of motion. The present paper describes the movements of a number of fish as recorded photographically against a scaled background. Unless otherwise stated the background is ruled into 3-inch squares and the time interval between successive photographs is approximately 0·04 seconds; the photographs are mounted so as to displace the fish to a uniform distance along the horizontal axis of the background.

In all the fish examined (Eel, Butterfish, Dogfish, Rudd, Whiting and Goldfish), the same fundamental mechanism is employed for effecting a change in the direction of motion, viz., the propagation of a wave of muscular contraction from the anterior end of the body towards the tail. This wave is of a similar nature to that which effects normal locomotion (see Gray, 1933, *a*), but is of abnormally large amplitude. The fish always turns towards the side of the body down which the wave is passing. The passage of the waves can be very clearly seen in the photographs of the Butterfish, Plate 3, and of the Dogfish, Plate 4. In order to understand the way in which the direction of motion of the fish is changed by the propagation of such muscular waves, it is essential to consider, not so much the changes which the waves cause in the form of the fish as the movements which the waves induce in each part of the body relative to the fixed environment of the fish. In many ways the essential features of a turning movement are illustrated most readily by such types as the Whiting and the Goldfish, but from a theoretical standpoint, the phenomena are best illustrated, in the first instance, by anguilliform types such as the Eel and the Butterfish—for it is in these forms that the propagated muscular waves are most obvious, and where movement is carried out efficiently without the specialized caudal fin, which is of fundamental importance in other types.

Plate 3 shows a Butterfish (*Centronotus gunnellus*) turning to the right through an angle of approximately 30°. The passage of the wave is marked on each of the figs. 2–15, Plate 3, by a black dot. The original direction of motion of the fish is marked by the dotted line which is parallel to the lines marked on the background behind the fish, the subsequent direction of motion is marked by the dotted line which diverges to the right at an angle of approximately 30°. From figs. 2–8, Plate 3, it can be seen that successive regions of

the body move to the right of the original axis of motion until they lie along the new path of motion. Having reached this position they remain aligned along this axis. The effect is clearly due to the successive contractions and relaxations of each region of the body. The wave of contraction starts near the paired appendages and deflects the head of the fish to the right—this is followed by two simultaneous events (1) the relaxation of the contracted muscles, (2) the contraction of the muscles lying just posterior to the original point of contraction. These two subsequent processes tend to move the anterior end of the animal in opposite directions and as they are equally effective they cause no resultant change in the angular position of the anterior end of the body. Until approximately one-half of the fish has moved to its position on the new axis of motion, the point of maximum contraction remains situated on or near the original axis of motion and is not displaced during contraction, figs. 2–8 inclusive, Plate 3. From this point onwards, however, the muscular wave begins to affect the regions of the body lying posteriorly to the point of maximum contraction instead of the regions lying anteriorly to this point. In figs. 11–15, Plate 3, it can be seen that the turning movements are being carried out by a rotation of the posterior end of the animal towards the left, while the anterior region of the body remains practically unaffected. During the passage of the wave over the first half of the body, the head end is rotating about the hind end of the body and during the second half of its passage the tail is rotating about the anterior end of the fish. Between these two conditions, figs. 9–12, Plate 3, the head of the fish and the tip of the tail are only very slightly displaced in space, and the greater part of the displacement is being effected by the central region of the body.

The effect of a single wave of contraction can be summarized as follows. Until the wave has passed over half the length of the fish, each anterior region of the body is deflected in turn to the right whilst the posterior half of the body remains unaffected—during the second half of its course, however, the muscular wave operates on the posterior half of the body and deflects it in the opposite direction. This series of events is obviously controlled by purely mechanical conditions. Each successive contraction tends to deflect both the head and tail ends of the fish towards the right, but the actual amount of deflection carried out by each of the two ends depends on the position of the contracting muscles. Contraction begins at the anterior end of the body and consequently the force required to turn the anterior end to the right is small compared with the force required to turn the long posterior end in the same direction at an equal velocity—hence the angular displacement of the head is very large

compared with that of the region of the body lying posteriorly to the contracting muscles. In other words, the body acts as a reasonably steady fulcrum on which the head turns. When the contraction has passed to the hind end of the body the reverse is the case, and the tail now turns on the anterior region of the body, which now acts as a fulcrum. Between these two positions lies the phase where the central part of the body moves without effecting any marked change in the angular displacement of the head or of the tail, figs. 10–12, Plate 3. During this phase the force required to displace the central region of the flexible body is less than that required to move either the head or the tail.

The whole of these movements are of essentially the same nature as those which effect the normal locomotion of the fish, except that the amplitude of the muscular wave is increased and its velocity of propagation is decreased. This can be seen in Plate 3 by comparing the " turning " wave with that which precedes it on the left side (see figs. 1–7, Plate 3) and the one which succeeds it on the left side (see figs. 13–19, Plate 3)—the latter waves being marked by small crosses.

Essentially the same phenomena can be seen in the Dogfish, Plate 4, where the wave of muscular contraction starts about the level of the pectoral fins. The dotted lines ruled on the figures represent fixed axes (on the background of the tank) which are approximately parallel to the original and final directions of motion of the fish. By reference to these fixed axes it can be seen that the change in direction of motion is also carried out in three stages, (1) the head of the fish is deflected to the right, figs. 3–11, Plate 4, without any significant displacement of the trunk of the body; (2) the central region of the body (in the neighbourhood of the pelvic fins) is deflected to the left of the original axis whilst the head and tail are displaced to a comparatively minor extent, figs. 11–23, Plate 4 ; (3) the tail of the fish is deflected sharply to the left leaving the head and trunk comparatively undisturbed, figs. 25–35, Plate 4. It will be noticed that the tip of the tail remains close to the original axis of motion, figs. 1–23, Plate 4, until the wave of contraction has passed almost along the whole length of the fish, and that the tail only begins to align itself along the new axis when the process of relaxation has reached the level of the pelvic fins. The whole succession of events is essentially similar to that in the Butterfish, although the proportional displacements of the three regions of the body differ somewhat; in the Dogfish the movement of the central regions of the trunk are much more obvious.

Neither in the Butterfish nor in the Eel does the caudal fin appear to play

any essential rôle in the process of turning, but in fish with relatively short bodies (Rudd, Whiting, and Goldfish) the conditions are different. Plate 5, A, shows a Rudd turning to the right through an angle of approximately 80° within a period of 0·3 second. As before, a wave of muscular contraction starts about the level of the pectoral fins and passes along the right side. In figs. 1–4, Plate 5, A, it can be seen that the movements relative to the background are all confined to the anterior region of the body—the tail remaining along the original axis. From this point onwards the anterior region of the body remains relatively stationary and the hind end of the body completes the movement. Plate 5, B, shows very clearly that the normal turning power of the Rudd is dependent on the presence of the caudal fin. The fish is the same as that shown in Plate 5, A, but is turning to the left after amputation of the caudal fin. The amplitude of the muscular contraction is practically the same in the two cases, but it can be seen that instead of moving through an angle of 80°, the fish has only moved through about 35°; the reason is obvious from photographs 3–5. In the absence of the caudal fin, the head and the tail of the fish begin to move simultaneously to the left of the original axis—whereas in the presence of the fin, the tail remains stationary and the head alone moves (see fig. 4, Plate 5 in both series, and fig. 1). Since the curvature of the body is the same in the two cases, it follows that the angular displacement of the head (relative to the background) is greatly decreased by the amputation of the fin. Precisely similar observations have been made with the Whiting and the Roach, and there can be no doubt that one essential function of the caudal fin is to keep the hind end of the body stationary in the water whilst the muscular contractions of the body are turning the head and anterior end of the body towards the new direction of movement. The maximum change in direction of the fish cannot exceed the angular displacement of the head through the medium, and this can only be at a maximum if the hind end of the body is kept stationary during the initial phases of the movement.

It is obvious that the function of the tail fin as a fulcrum on which the head of the fish is able to be turned is, in the case of a fish with a long flexible body (*e.g.*, Dogfish), performed by the body itself. The larger the head is relative to the muscular trunk and tail of the body, the greater is the necessity for a caudal fin. The latter conditions are well marked in the common Goldfish which appears—when seen in a tank—to turn by a sudden flexure of the tail towards the side to which the animal turns. Actually, the movements of the Goldfish differ only in degree from those of the Rudd or the Whiting. Plate 6

shows a Goldfish with a comparatively wide and deep body changing direction to the right through an angle of about 80°. It can be seen that the tail does not, in fact, move to any marked extent until the head has almost completed the whole turn—and then, as in other fish, the tail swings into position by movement to the left (see also fig. 2). The Goldfish shows, however, that when the anterior region of the body is large and comparatively rigid, this region pivots about a point which is not far removed from its centre of gravity. In

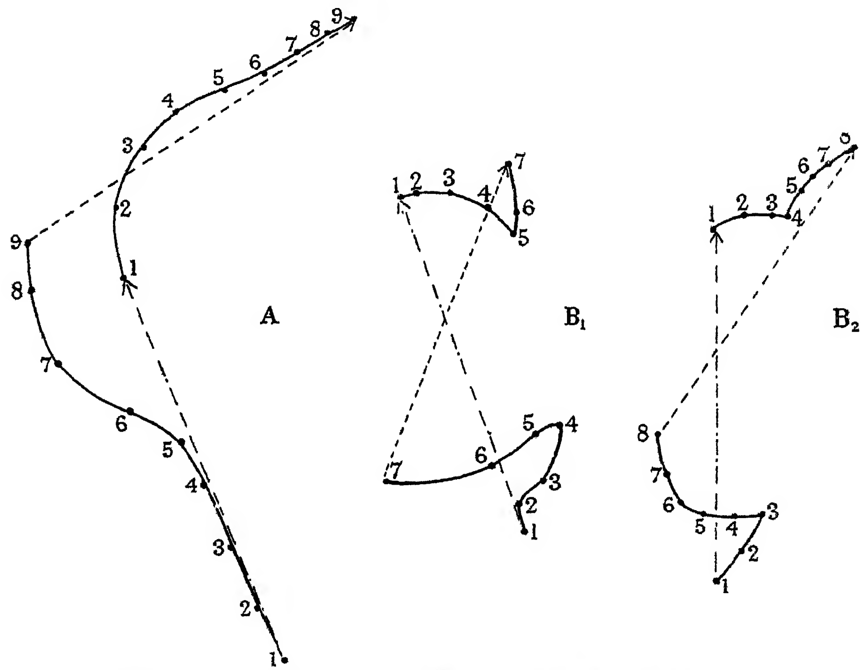

FIG. 1.—A. Track of head and tip of tail of a Rudd when making a normal turn of about 90°. Note that the tip of the tail remains along the original axis of movement until the head has reached its new axis. B_1 and B_2. The same fish as in fig. A, after amputation of the caudal fin. Note in both cases that the tail is deflected from the original axis almost as soon as the head itself begins to turn. The dotted lines in the figure indicate the positions of the fish immediately before and after the turning movement.

Plate 6, A, the pivot is marked on the back of the fish by a black dot and in figs. 1–4, Plate 6, it can be seen that this point remains practically stationary and the turn is effected by rotating the anterior end of the fish about this point (the head moves to the right and the hind end of the trunk, marked by a cross, moves to the left). As might be expected, amputation of the caudal fin of the Goldfish reduces the ability of the fish to change its direction of motion

to an even greater extent than for the Rudd or Whiting, Plate 6, B and C and fig. 2 B.

The rôle of the caudal fin during turning movements must clearly be considered in association with the form of the whole body.* The essential condition for rapid changes in direction is that the head should be able to turn without displacement of the hind end of the body. This condition is fulfilled as long as the resistance to transverse motion of the head is low compared with

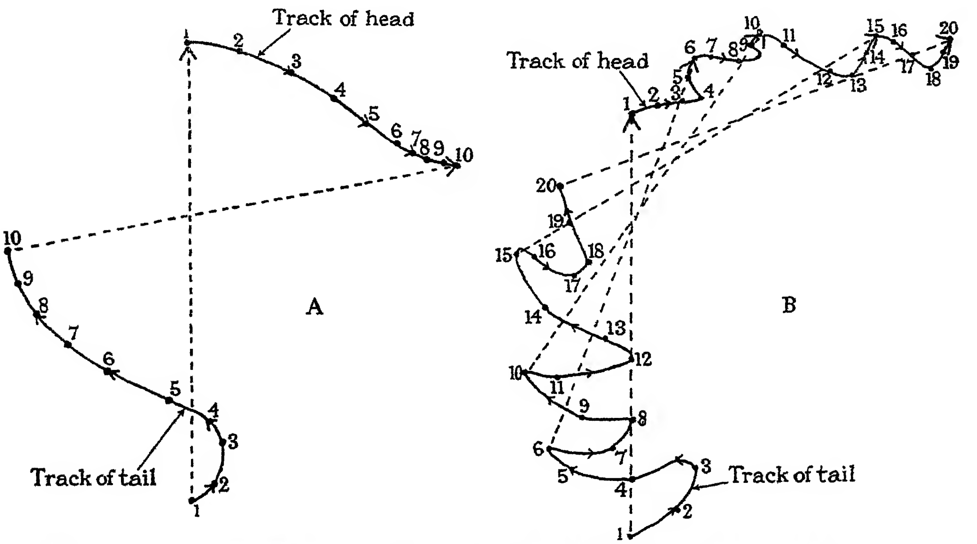

FIG. 2.—A. Track of head and tip of tail of an intact Goldfish turning to the right through about 80°. Note that the displacement of the tail is slight until the head has turned through a considerable angle, and that the whole turn is carried out by one flexure of the body. B. Track of the head and stump of the tail of the same Goldfish when turning to the right through about 80°, after amputation of the caudal fin. Note that four successive flexions are required (the dotted lines show the position of the body after each). Note that whenever the head begins to move to the right, the tail also moves in the same direction.

that of the region of the body behind the point of contraction. In all fish the anterior region of the body is free from unpaired fins which would, if present,

* Among the fish mentioned there is no evidence that the tail fin is used as a rudder, but in such types as the marine stickleback (*Gasterosteus vulgaris*) where locomotion is mainly effected by the pectoral fins, the muscular tail is relatively rigid and inert, and turning movements are effected by flexure of the tail-fin towards the side to which the animal turns. The turning effect is similar to that produced by the rudder of a ship and unlike that of a transmitted muscular wave, it can only be exercised when the whole fish is in motion; the rate of turning is very much less than that seen in fishes which turn by means of their somatic musculature.

increase the resistance of the head to lateral movements. The presence of a fin at the extremity of the tail automatically increases the resistance of the hind end of the body to a maximum extent, although a similar effect will be exercised by any other fin situated between the point of muscular contraction and the posterior end of the body. Since the caudal fin is situated at the greatest distance from the point of contraction it follows that its effect on the motion of the body must be the most significant. The greater the resistance of the rigid head end of the body, and the shorter the muscular body behind this head region, the greater is the pressure which must be exerted by the tail fin when the fish is turning. All the dorsal and ventral fins of a fish must, to some extent, affect the precise nature of the turning movements, and it is significant that these fins are typically restricted to the posterior regions of the body.

It is, however, important to notice that the function of the caudal and other unpaired fins is not entirely restricted to that which is exercised during turning movements. The type of muscular wave which induces a change in direction is simply an exaggeration of that characteristic of normal swimming movements, and it therefore follows that the caudal fin must affect these movements also. It is commonly assumed that the caudal fin plays an important rôle in the propulsion of the fish through the water. This is probably true for such fish as the Mackerel, but for Whiting and Rudd, the rate of movement at reasonably slow speeds is not significantly reduced by amputation of the caudal fin. Removal of the fin affects the movements of the fish in a perfectly definite way (see Gray, 1933, *b*) but it does not greatly influence the speed of propulsion. This aspect of the problem will be discussed elsewhere; this paper is only concerned with one of the effects which the fin exercises by virtue of its resistance to transverse movement.

From the facts described above, it is clear that the differences observed between the turning movements of an Eel on the one hand, and of a Goldfish (with and without a caudal fin) on the other, are entirely of a mechanical nature, for the caudal fin of the Goldfish is playing an essential rôle solely on account of the comparative shortness and rigidity of the rest of the body. It is shown elsewhere (Gray, 1933, *b*) that a similar rôle is exerted by the fin during the normal locomotion of the fish, and it is therefore clear that the evolution of the caudal fin must, from a functional point of view, be considered in conjunction with the evolution of the body of the fish as a whole. If a general trend of evolution involved a relative shortening of the long axis of the body, this change must have been accompanied by the development of an

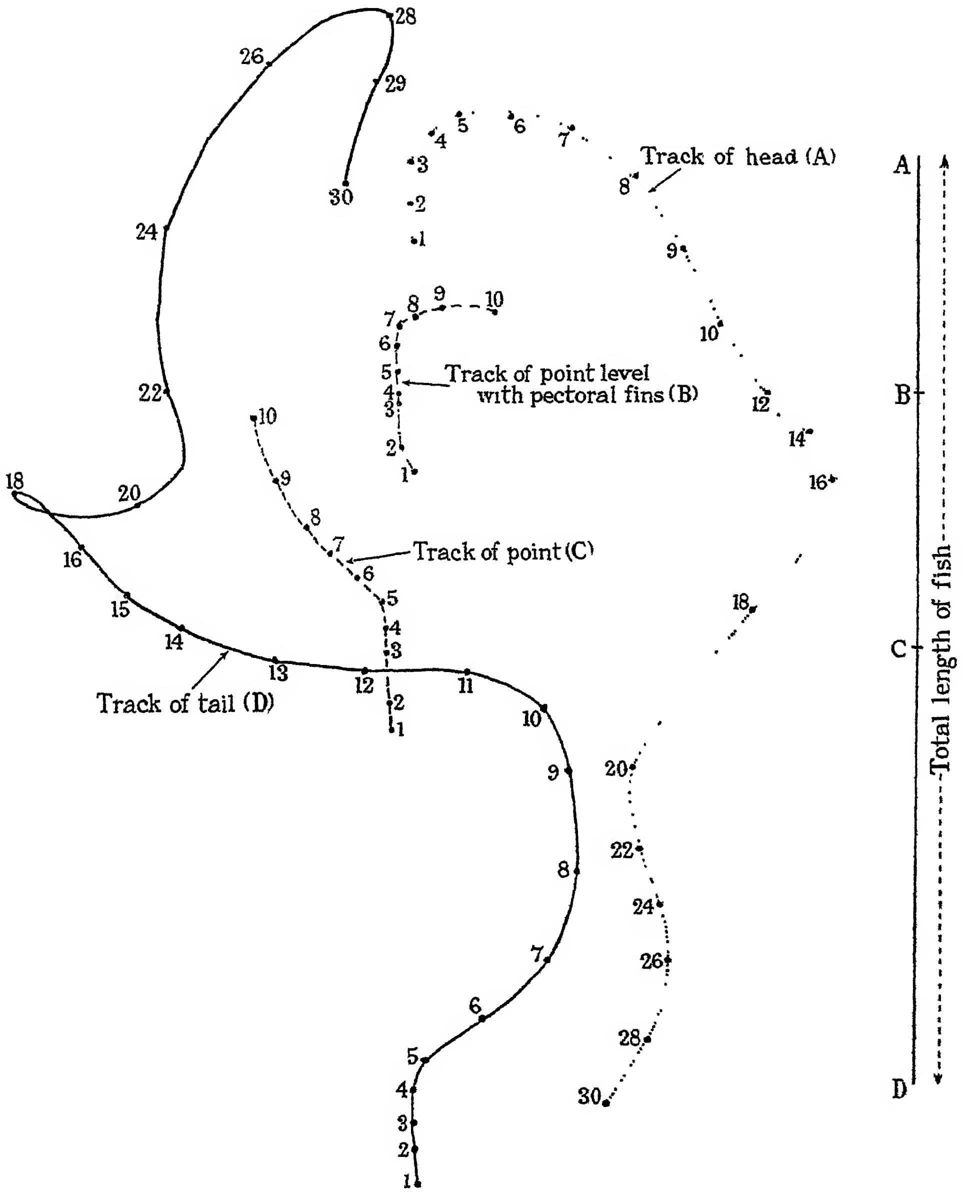

FIG. 3.—Track of four points on the body of a Whiting when turning through approximately 180°. The whole process occupied about 1½ seconds and represents the effect of two separate muscular waves. For points B and C only the tracks during the first ½ second are shown.

expanded caudal fin restricted to the tip of the tail, otherwise rapid turning movements and normal smooth progression (see Gray, 1933, *b*) would have become impossible; any increase in the rigidity of the anterior end of the body accentuated the rôle of the caudal fin. It is perhaps significant to note that the proportions of a typical pelagic teleost, approximating as they do to those of a stream-lined form, are such that a caudal fin is essential even if this fin were to play no significant rôle as a propellant surface. On the other hand, fish without well-defined caudal fins must have elongated flexible bodies.

In comparison with the rudder of a ship, the turning effect of the somatic muscles of a fish is strikingly effective. It can be seen in Plate 7 and fig. 3 that a Whiting can readily turn through 180° within a circle whose diameter is not greater than the length of the fish's body.

Summary.

(1) In all the fish examined, a change in the direction of motion is effected by propagating an abnormally large wave of muscular contraction down the side of the body towards which the animal turns.

(2) During the first phase of the movement, the anterior end of the fish turns on the posterior end which remains relatively stationary in position. During the final phase of the movement, the posterior end of the body moves towards the new axis of movement and during this process the anterior end of the animal remains relatively stationary.

(3) Except in fish with long and flexible bodies, the caudal fin plays an essential rôle during turning movements for it enables the posterior end of the body to act as a fulcrum on which the head is able to move through the water.

(4) A typical pelagic fish such as a Whiting can turn through 180° within a circle whose diameter is not greater than the length of the whole fish.

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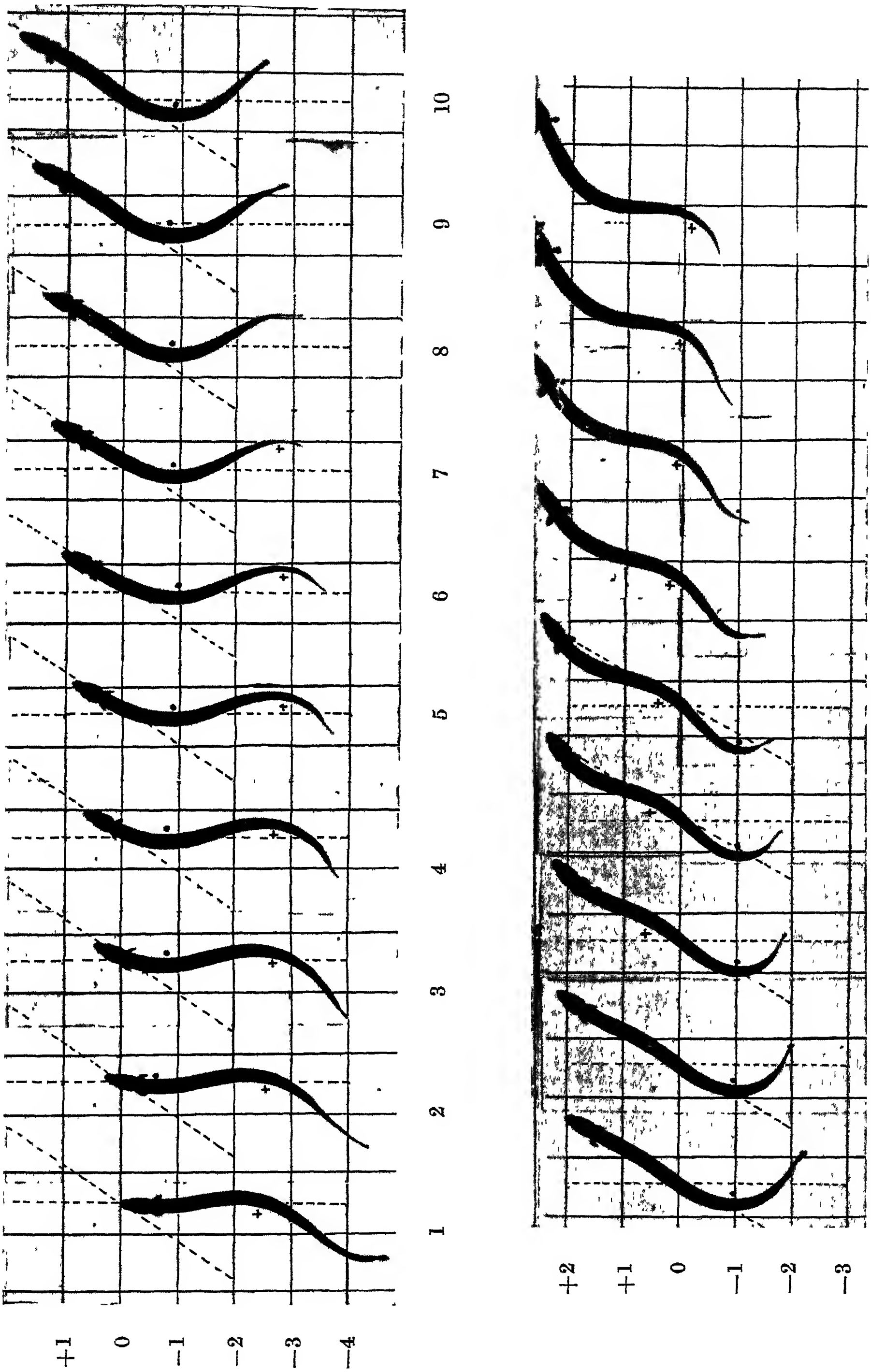
PLATE 3.

Butterfish (*Centronotus gunnellus*) turning to the right through an angle of approximately 30°. The "turning" wave is marked by black dots; the normal propulsive waves are marked by crosses. The turn is carried out by the movement of successive regions of the anterior half of the body to the right, and subsequently of the posterior half of the body to the left.



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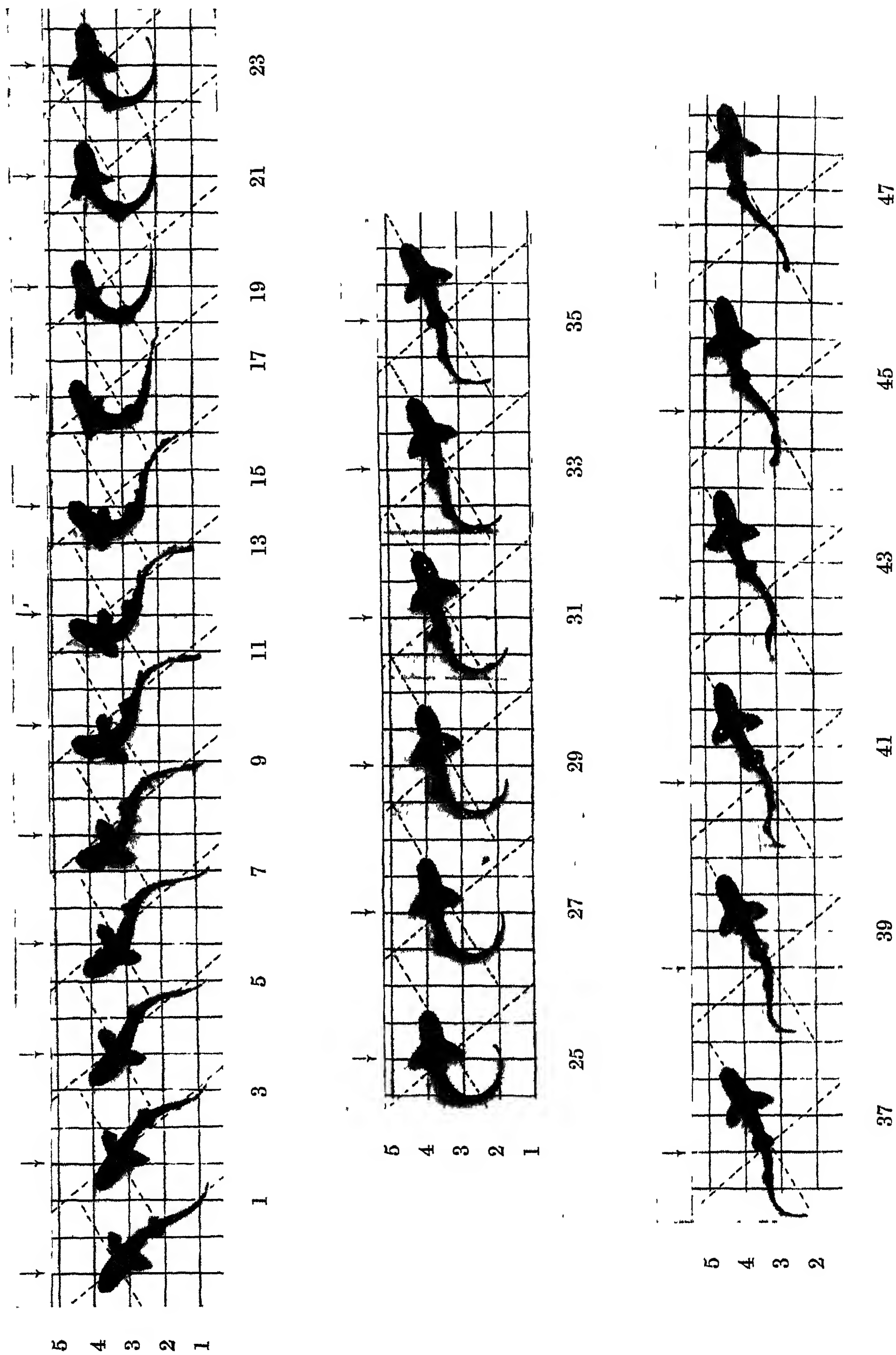

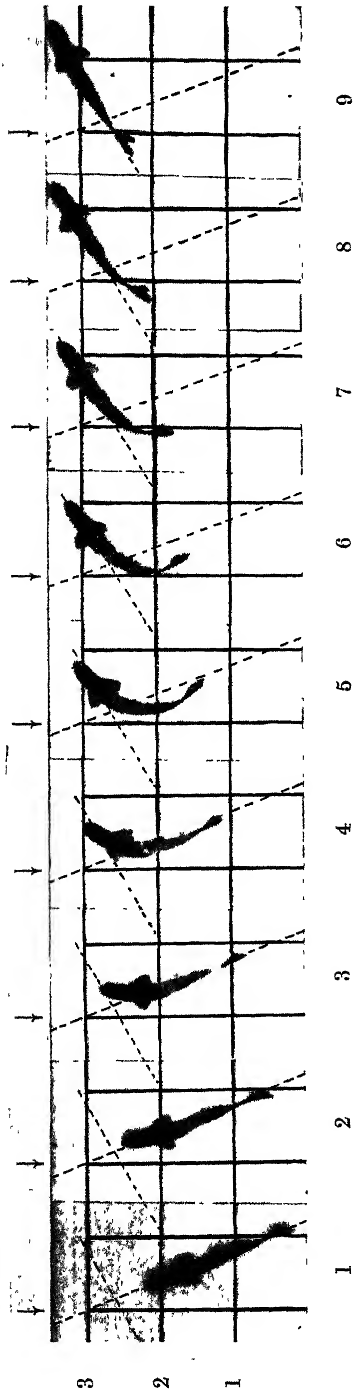

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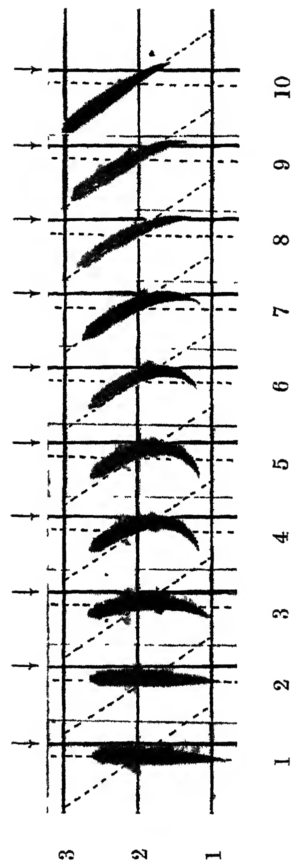

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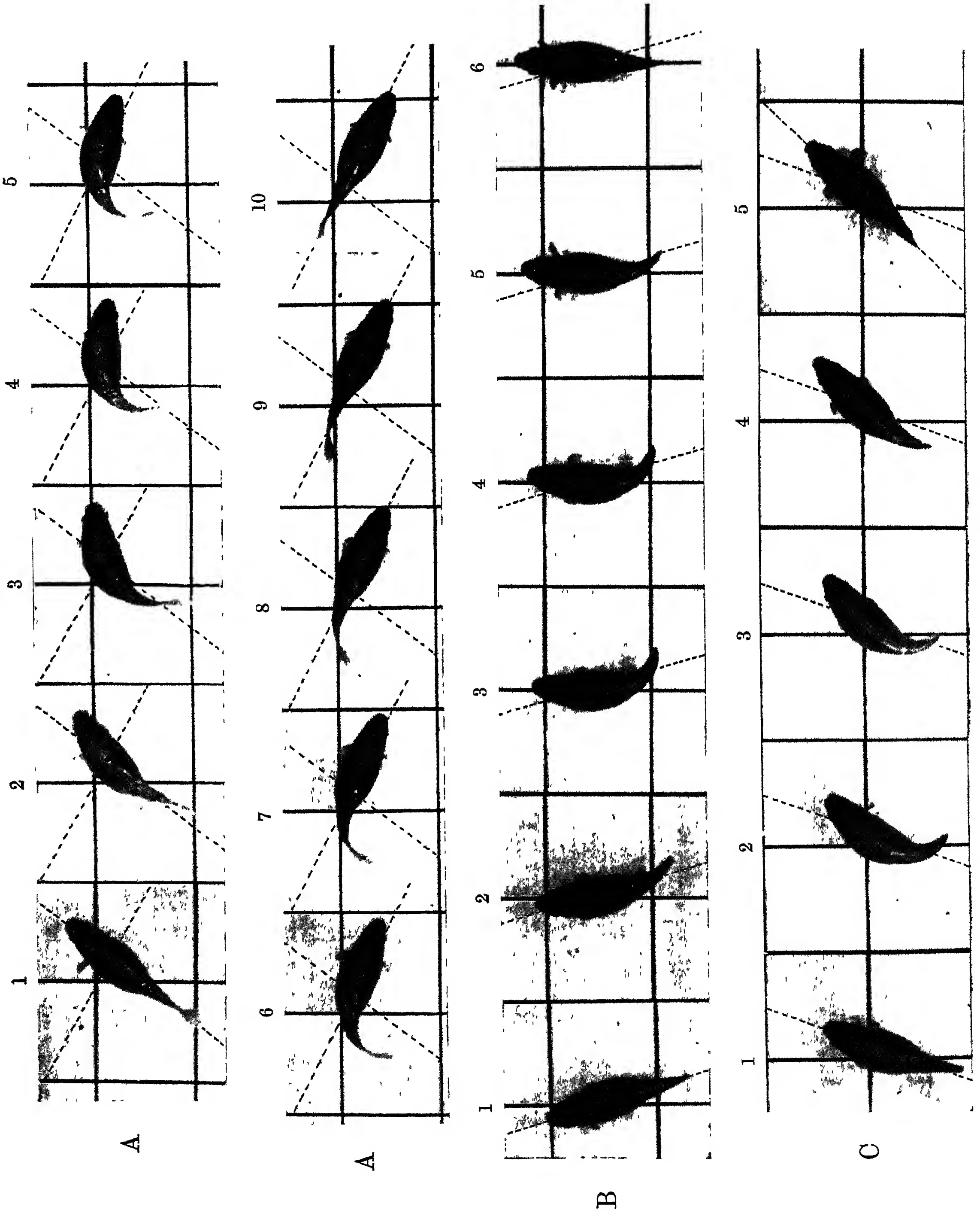
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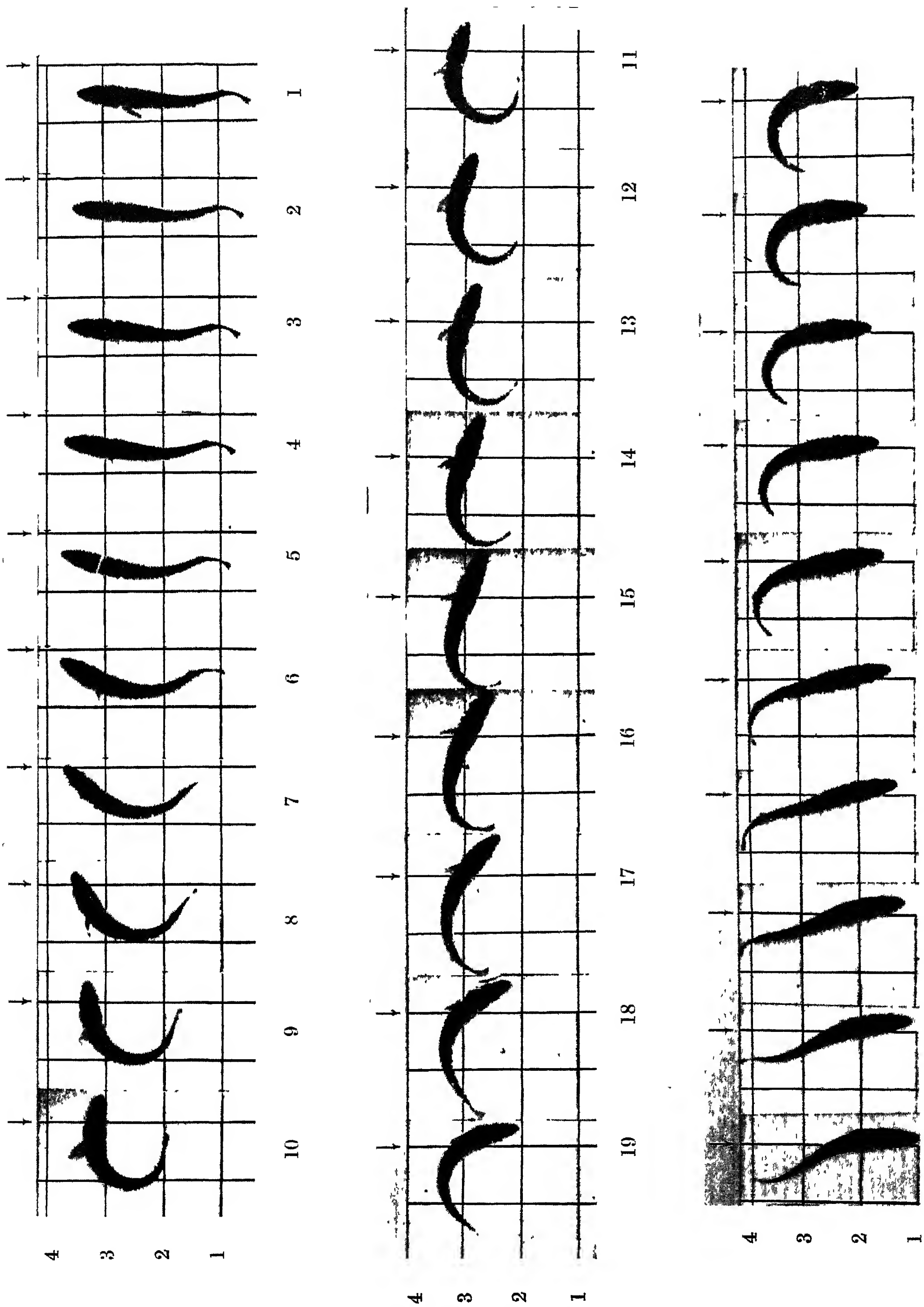
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PLATE 4.

Dogfish (*Scyllium canicula*) turning to the right through rather more than 90°. The interval between each successive photograph was 0·04 second, but only every alternate photograph is shown. The distance between the arrows above each row of photographs shows the extent to which the photographs have been displaced along a horizontal axis. Note that the tail is not greatly displaced from its original axis until the anterior end of the body has almost completed the turn.

PLATE 5.

Rudd (*Cyprinus erythrophthalmus*): A. Normal fish turning to the right through approximately 80°. Note that the anterior end of the fish moves without seriously displacing the position of the hind end of the body. B. The same fish turning to the left after amputation of the tail fin. Note that as soon as the head moves to the left, the tail also moves to the left (photographs 3 and 4), thereby reducing the angular displacement of the head through the water. The movements of the tail relative to the head are practically the same as those shown in the intact fish in series A.

PLATE 6.

A. Goldfish (*Cyprinus auratus*) turning to the right through about 80°. Note that the anterior end of the body pivots round a point marked by the black dot, and that this movement does not markedly displace the tail fin. B and C. The same fish as in A after amputation of the caudal fin. Note that the amplitude of the movement of the tail relative to the head is practically unchanged by the operation but the ability to turn the fish is very greatly reduced.

PLATE 7.

Whiting (*Gadus merlangus*) turning to the right through nearly 180°. The turn was carried out by two successive contractile waves, the second of which begins about photograph 19. The fish was about 9 inches long and turned in an area of approximately 9 inches by 9 inches.

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The Mechanism of the Action of the Growth Substance of Plants.

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1. Introduction.

It was first suggested by Sachs that stimulating substances of the type which would now be called hormones play an important part in cell growth. Experimental evidence followed much later along two lines, (*a*) the discovery by Haberlandt (1921) of a substance produced in wounded tissue, stimulating cell division, and (*b*) the gradual accumulation of evidence from many workers of the existence of a substance present in growing plants which stimulates growth by increase in cell size. It is with certain aspects of this latter phenomenon with which we are concerned. Since the earlier extensive literature has been reviewed by Stark (1926) and the more recent literature by Went (1928) and Nielsen (1930), it will be sufficient here to summarize briefly the properties of this growth substance in relation to plant growth, so far as they have been determined.

Although the growth substance has been shown to be non-specific, we have limited ourselves in this work to consideration of the phenomena occuring in Avena coleoptiles. In these coleoptiles, at the age used for experiment, cell divisions do not occur, and growth takes place by extension only. On this account they have been the principal objects studied.

The facts which have been established for Avena coleoptiles are briefly as follows.

(*a*) The growth substance is produced in the tip of the coleoptile and diffuses downwards towards the base.

(*b*) If the tip is removed, the topmost remaining region commences to produce the substance, after the lapse of a definite time.

(*c*) The response of the coleoptile to phototropic and geotropic stimuli is conditioned by the presence of growth substance. After decapitation, the ability of the uppermost zones to react to geotropic and phototropic stimuli

is lost, but subsequently returns at the same time as the new growth substance is produced.

(*d*) If, after decapitation and re-appearance of growth substance in the upper zones, these zones are also removed, the growth of the coleoptile falls practically to zero.

(*e*) Application of growth substance, obtained from the tips, to the lower zones of old coleoptiles which have stopped growing, causes the recommencement of growth.

From the three experimental facts, (*c*), (*d*), and (*e*), it has been fairly concluded that the growth substance is not merely an accelerator of growth, but that without it no growth can occur (" Ohne Wuchstoff kein Wachstum " (Went, 1928)).

(*f*) The lower zones of a coleoptile, since they are still growing, presumably are supplied with growth substance, yet it cannot be recovered from them. The growth substance, therefore, disappears continuously during growth.

(*g*) The movement of growth substance in the coleoptile is strictly a polar phenomenon (Van der Wey, 1932). In inverted coleoptile cylinders, no downward transport occurs. In coleoptiles placed horizontally the growth substance passes to the lower side and there increases the growth, causing an upward curvature.

(*h*) The application of growth substance to decapitated coleoptiles results in an increase in the plasticity of the coleoptiles ; their irreversible extensibility under tension is greater, and they can be permanently curved by a slight lateral force.

(*i*) Studies of growth substance show that it is not an enzyme, but is a thermostable, lipoid-soluble substance, free of nitrogen. It has a strong acidity (p_K 4·75) and high molecular weight (about 341). It is active in very high dilution, so that the amounts of it actually produced in coleoptile tips are excessively small.

(*j*) A growth substance is produced in relatively large quantities by the growth of moulds and bacteria, from which highly active concentrates may be obtained. The same, or a similar substance, is widely distributed among higher plants.

The above facts, gathered in the last ten years, can be summarized in the statement that the growth substance is a well-characterized compound whose properties and behaviour determine the growth and tropisms of *Avena* coleoptiles, and probably also the growth and tropisms of other plants.

From these data, however, little may be inferred as to the actual mechanism of the reaction by which the growth substance induces growth through elongation of cells. It is with this mechanism that we are concerned in this work.

Sachs, to whom is due the first modern theory of growth, believed that increase in cell size is the result of stretching the cell wall under the influence of turgor pressure. As the wall stretches, however, the particles of which it is composed become farther apart and new ones are introduced. When these new particles are laid down, the tension upon the wall, owing to its stretching, is decreased and the process may be repeated as long as sufficient turgor pressure is exerted. Opinions have differed as to whether the initiation of growth is caused by an increase in turgor pressure or by an increase in the elasticity or plasticity of the wall, and also as to whether the laying down of new particles is itself a necessary accompaniment of this growth.

Overbeck (1926), by plasmolytic methods, was able to separate the elastic and plastic portions of geotropic curvatures in roots, and to show that the "fixation" of the elastic portion of curvature is a secondary process, which may be suppressed by low temperature. The work of Heyn (1931) has contributed extensively to an understanding of the growth process. He attempted to determine which properties of the coleoptiles change as a result of the presence of growth substance, and to reach in this way a decision as to what factors primarily cause, and what factors result from, cell elongation. Heyn has shown that (*a*) growth substance increases the plasticity of the cell wall, and to a slight extent also the elasticity; (*b*) this increase in plasticity is the primary factor permitting cell elongation; (*c*) after increase in wall plasticity, the force causing elongation is the normal turgor pressure; (*d*) the changes in elasticity accompanying the action of growth substance are of small magnitude and probably of little importance.

Since these changes were obtained with plasmolyzed material, there is no question but that they represent a real cell-wall phenomenon. It is therefore unlikely that the primary action of the growth substance is one of a change in osmotic pressure or in the water permeability of the cell membrane. The recent experiments of Söding (1932), which show that the first stages of curvature are not reversed by plasmolysis, confirm this view. The remaining possibilities are: (*a*) the growth substance performs its function in the protoplasm, and that the protoplasm itself, in some unspecified way, influences either the plasticity of the wall or the deposition of cellulose; (*b*) the growth substance acts directly upon the cell wall, either (1) by catalyzing the production

of new wall material, (2) by an effect on the properties of the wall micelles, or (3) by an effect upon the intermicellar fluid.

A direct action upon the cell wall would be likely to involve a stoichiometric relation between the growth substance and the wall constituents, and we have, therefore, in this study determined whether or not any simple relation exists between the number of molecules in a given amount of cell wall and the number of molecules of growth substance necessary to produce it. This involves the reasonable assumption that the material formed by the action of the applied growth substance is of the same composition as that of the whole coleoptile, and this assumption is supported by evidence given in Section 4. The experimental work is divided into five heads corresponding to the pieces of information required in order to calculate the desired relation.

These heads are as follows :—

(1) The amount of growth substance entering the plant under the experimental conditions.

(2) The amount of growth produced by a given amount of growth substance.

(3) The actual increase in weight of cell wall involved in a given amount of growth.

(4) The composition of the cell wall and hence the increase of each constituent for a given amount of growth.

(5) The increase in volume of cell wall for a given amount of growth. This is an independent method of arriving at the result given by (3).

2. Apparatus and Materials.

Growth Measurements.

The growth measurements were carried out with an automatically controlled motion picture camera. The films, after development, were projected with a final enlargement of 100 times upon a screen, on which the images of the plants could be measured with considerable accuracy. The Leitz camera was operated with a " De Bouter intermittent clinostat," which in turn was operated by an electric clock. A general description of this type of clinostat is given by F. A. F. C. Went (1929). The two plants which were photographed upon each film were placed about 1 metre from the camera with their roots in water. Beside each plant was placed a millimetre scale which was included in the photograph and from which the growth increments were measured.

Since the etiolated coleoptiles are very sensitive to light of wave-length shorter than 5300 A., the photographs were taken in orange light, using Wratten filter No. 24, which cuts off most of the light shorter than 5750 A. Eastman supersensitive panchromatic film was used with an exposure of 5 seconds.

The experiments were carried out in a constant temperature room at 27° C. The relative humidity was kept at 90 per cent., with a variation of not more than 3–4 per cent. Since the standard conditions for testing the activity of growth substance preparations by the curvature method used in this laboratory include a temperature of 25° C., a second room was kept at this temperature and the activity tests carried out in it.

Preparation of Growth Substance.

The growth substance was obtained from the large scale culture of the mould *Rhizopus suinus*, described by Thimann and Dolk (1933). The mould was grown upon a peptone-glucose culture medium, and care was taken to keep the conditions aerobic, since this greatly increases the yield of growth substance (Bonner, 1932). At the end of 10 days the mycelium was filtered off, the medium concentrated under reduced pressure to 1 per cent. of its volume, filtered, acidified, and extracted six to seven times with peroxide-free ether. The bulk of the ether was distilled off, the residue taken up in a small volume of water, boiled free of ether, and the solution chilled. Chilling causes the precipitation of a rather large quantity of oily material which was filtered off. The filtrate is a growth substance preparation of high activity, from which suitable dilutions may be made for each experiment. Its activity was of the order reached by Dolk and Thimann (1932), namely, about 10^{-6} mg. per plant unit. It is possible to work with these solutions, whose relative concentrations are accurately known, in a much more quantitative fashion than with the old method by which small amounts of growth substance were obtained by diffusion from coleoptile tips into agar.

Tests.

The tests of the activity of the growth substance preparations were carried out as described by Dolk and Thimann. This test depends upon the curvature produced in decapitated coleoptiles by the one-sided application of an agar block containing the growth substance. This curvature has been shown to be, within certain limits, proportional to the concentration of growth substance in the agar block (Went, 1928; Nielsen, 1930; Van der Wey, 1931). In

order to obtain consistent and comparable results, the conditions of temperature, humidity, and time relations must be carefully controlled.

3. Amount of Growth Substance entering the Plant.

Determinations.

It has recently been found (Thimann and Bonner, 1932) that the rate at which growth substance passes from solution in agar into the decapitated coleoptile is directly proportional to its concentration in the agar, and may be expressed by the equation

$$-\frac{dx}{dt}=\mathrm{K}\frac{x}{v},$$

where x is the amount in a block of volume v. From experiments in which agar blocks containing growth substance were removed, after having produced curvatures, and placed upon fresh decapitated coleoptiles, the rate constant of passage was determined. The fraction of the growth substance initially present which would pass out of blocks of different volumes was calculated from the value of this constant, and shown to agree with the experimental results. For blocks of our standard size this fraction is 15 per cent. It was also shown that for blocks of small volume a very large fraction of the growth substance initially present passes out, which accounts for the belief, held by earlier investigators, that all of the growth substance passes from block to coleoptile.

While the above work was concerned with blocks placed unilaterally upon coleoptiles, the present experiments are concerned with vertical growth in which blocks are placed flat upon the coleoptile stump. Since the area of contact is more than doubled, it might be expected that the proportion of growth substance passing into the plant in this case would also be more than doubled. For the evaluation of this proportion, large numbers of plants were decapitated and supplied with agar blocks containing a known amount of growth substance, precisely as was done later with the plants whose growth rates were to be measured. At the expiration of 105 minutes, the blocks were removed, placed unilaterally upon decapitated test plants, and the resulting curvatures measured. A curvature θ is, as pointed out by Thimann and Bonner, proportional to the concentration of growth substance in the agar block. Since blocks were made from a growth substance solution of known strength, they would, if placed upon test plants, give an initial curvature θ_0. From the curvaure θ_1 given by the second test plant, the amount of growth substance in

the block at the beginning of the second test is also known. Hence, the fraction of the initial quantity of growth substance which passed out of the block while it remained flat upon the coleoptile is given by $\theta_0 - \theta_1/\theta_0$.

Table I gives a series of determinations of θ_1, and of this fraction, for different values of θ_0. The mean value of the fraction passing out is 34 per cent.

Table I.—Percentage of Growth Substance Passing into Plant from Blocks flat upon the Coleoptile Stump.

Units applied (Initial possible curvature) θ_0.	Units remaining (Curvature on second application) θ_1.	Percentage passed out of block $\frac{\theta_0 - \theta_1}{\theta_0} . 100.$	Number of plants.
14·4	9·0	37·5	17
14·4	9·8	32·0	15
19·2	13·0	32·3	21
19·2	12·0	37·5	16
Mean percentage passed out of block		34	

Units.

The fact that, of a given amount of growth substance applied to the plant, only a small proportion actually enters, necessitates reconsideration of the principles underlying the idea of a unit. As defined by Dolk and Thimann, the unit is that amount of growth substance which, when present in 1 c.c. of solution, will give after mixing with 1 c.c. of 3 per cent. agar and making into blocks of volume 10·7 mm.^{3}, a curvature of 1° in the standard Avena coleoptile. The actual amount of growth substance in the block (one "plant unit") is thus 10·7/2000 of the amount present in 1 c.c., and as mentioned above, only 15 per cent. of this actually enters the plant under the standard conditions of curvature test, so that the final growth response is produced by 0·0008 of the amount present in 1 c.c.

Although the unit adopted by Kögl and Haagen-Smit (1931) is defined in much the same way as above, the agar blocks used by these workers are considerably smaller than ours, having a volume of 2 mm.^{3} Other conditions being equal, 58 per cent. of the growth substance would pass out of these blocks (see Thimann and Bonner, Table IV), and hence the final growth response would in this case be produced by 0·0032 of the amount present in 1 c.c. of solution. Our units are thus about four times those of the Dutch workers. Since, however, they have used the double decapitation method of Van der Wey, this relationship may not be exactly correct.

Boysen-Jensen (1932) has defined a still larger quantity as a unit, taking the amount which, when present in 50 c.c., gives on mixing with 50 c.c. agar, a *d* value of 1 mm., which, if the coleoptiles are assumed to be 1·5 mm. in thickness, is equal to 38° (see also Nielsen, 1930). This method of measuring the curvature in terms of the difference in length of the two sides has been previously discussed (Dolk and Thimann). There are, therefore, two types of influences to be considered: those causing variation in the actual curvatures obtained for a given amount of growth substance applied, and those resulting from different methods of measuring these curvatures. The above considerations do not seem to us to invalidate in any way the conception of a unit; they merely emphasize that, in order to obtain comparable results, the technique must be defined and rigidly maintained in all its details.

4. The Amount of Growth Corresponding to a given Amount of Growth Substance.

Although it has been shown that the relation of response of coleoptiles to unilateral application of growth substance is linear over a certain range, the response to symmetrical application has not been quantitatively followed. The response to different amounts of growth substance contained in agar blocks laid flat upon coleoptiles was therefore measured in order to determine whether the relationship is also linear under these circumstances, and, if so, over what range.

Preparation of Plants.

The Avena plants which were used in these experiments were of the pure line "Siegeshafer," which was kindly supplied by Dr. Åkerman of Svalöv. The method of growing the plants was that described by Went (1928). After removal of the husks the seeds were soaked 3 hours in water. They were then allowed to germinate for 24 hours in the 25° C. dark room and at the expiration of this time were planted in glass holders with their roots in water.

After 2 days from the time of planting they reached a length of 2–3 cm. Coleoptiles exhibit their maximum rate of growth when they are about 3 cm. long (Königsberger, 1922) and the rate of growth is not greatly different for plants a little shorter. Since for these experiments it is only necessary to choose plants of similar growth rates, straight plants of lengths between the above limits were chosen.

With the aid of a sharp blade the tip of the coleoptile was cut at a point 5–6 mm. from its apex, the first leaf drawn out until only 2–3 mm. remained and cut off flush with the top of the coleoptile. This piece of leaf was allowed to remain, in order to prevent the gelatin from flowing into the cylinder. The plants were then removed to the dark room containing the apparatus, and placed in position beside the millimetre scales. If the plants were to be supplied with growth substance this was done by fastening upon the stump, with 15 per cent. gelatin, an agar block containing growth substance in the desired concentration. The clinostat was next started and the first picture taken as quickly as possible, which was in general 2–3 minutes after decapitation.

Two pictures, separated by a 2-minute interval, were taken each 15 minutes. The values for the growth increments in the subsequent tables are obtained from the means of these two pictures.

Rate of Growth of Decapitated Coleoptiles.

The rate of growth of decapitated coleoptiles, unsupplied with growth substance from an outside source, was first determined.

Fig. 1 gives graphically the results obtained from the mean values of six plants, the rate of growth in millimetres per 15 minutes being plotted against

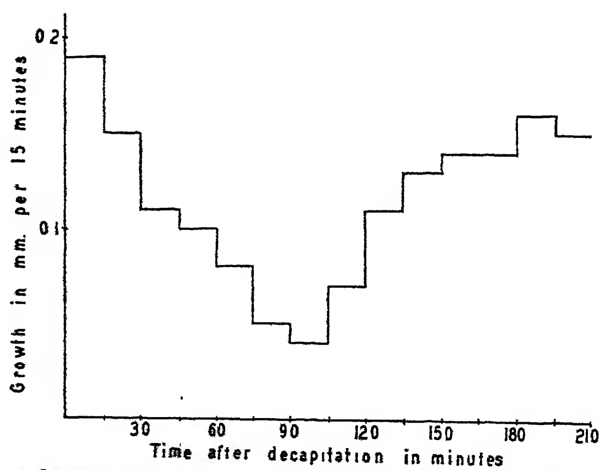

Fig. 1.—Growth rate of decapitated coleoptiles not supplied with growth substance.

time. The rate of growth may be seen to decrease steadily after decapitation for 105 minutes. After this time the rate increases rather sharply, and reaches a constant level which is, however, not so high as the original. An inspection of the actual data, Table II, reveals that the mean values, plotted in fig. 1,

Table II.—Growth Rates of Decapitated Coleoptiles Unsupplied with Growth Substance.

Plant No.		Time after decapitation in minutes. 15.	30.	45.	60.	75.	90.	105.	120.	135.	150.	165.	180.	195.	210.
II-1	Growth increments in mm. per 15 minutes.	0·12	0·21	0·10	0·14	0·07	0·09	0·01	0·15	0·20	0·19	0·19	0·20	0·10	
II-2		—	0·09	0·09	0·09	0·08	0·02	0·08	0·16	0·12	0·10	0·11	0·15	—	—
IV-1		—	0·12	0·04	0·04	0·03	0·03	0·04	0·00	0·01	0·11	0·16	—	—	—
IV-2		—	0·11	0·05	0·10	0·06	0·02	0·03	0·04	0·15	0·13	0·10	—	—	—
VI-1		0·23	0·23	0·22	0·09	0·13	0·04	0·06	0·04	0·02	0·10	0·10	—	—	—
VIII-1		0·20	0·12	0·13	0·17	0·11	0·14	0·04	0·03	0·09	0·09	0·16	0·07	0·16	0·15
Means	....	0·19	0·15	0·11	0·10	0·08	0·05	0·04	0·07	0·11	0·13	0·14	0·14	0·16	0·15

Table III.—Average Growth Rates of Coleoptiles under the Influence of different Amounts of Growth Substance.

Amount of g.s. in block.		Time after decapitation in minutes. 15.	30.	45.	60.	75.	90.	105.	120.	135.	150.	165.	180.	195.	210.	225.	240.	255.	270.	285.
0	Growth increments in mm. per 15 minutes.	0·19	0·15	0·11	0·10	0·08	0·05	0·04	0·07	0·11	0·13	0·14	0·14	0·16	0·15	—	—	—	—	—
9·9		0·26	0·15	0·16	0·14	0·12	0·11	0·13	0·13	0·09	0·09	0·08	0·07	—	—	—	—	—	—	—
14·5		0·21	0·22	0·22	0·21	0·20	0·20	0·21	0·19	0·13	0·01	0·07	0·06	0·08	0·09	0·09	0·11	0·11	—	—
19·3		0·27	0·25	0·25	0·21	0·24	0·25	0·26	0·23	0·19	0·17	0·14	0·12	0·10	0·09	0·09	0.10	0·10	—	—
29·0		0·31	0·31	0·29	0·29	0·27	0·26	0·29	0·30	0·26	0·25	0·23	0·18	0·13	0·11	0·12	0·11	0·11	0·11	—
39·5		0·31	0·33	0·31	0·30	0·31	0·33	0·33	0·32	0·32	0·27	0·22	0·20	0·16	0·13	0·11	—	—	—	—
43·5		0·28	0·28	0·28	0·30	0·30	0·32	0·34	0·31	0·36	0·34	0·28	0·25	0·19	0·15	0·12	0·12	0·12	—	—
58		0·35	0·32	0·35	0·36	0·37	0·40	0·42	0·37	0·42	0·42	0·33	0·30	0·23	0·20	0·14	0·12	0·12	0·13	—
79		0·33	0·41	0·33	0·37	0·36	0·36	0·47	0·45	0·32	0·35	0·28	0·31	0·31	0·23	0·15	0·11	0·09	—	—
116		0·34	0·33	0·36	0·37	0·38	0·39	0·42	0·40	0·39	0·39	0·39	0·31	0·26	0·24	0·17	0·13	0·11	0·10	0·10

do not accurately represent the growth of the individual plants. In general, the growth of the plants tends to drop gradually to a minimum and mounts quickly to the new constant level, but this is obscured in the mean, since the minima are somewhat spread out. This sudden physiological regeneration of a growth-substance-producing region in the stump will be considered in the next section. It is also an indication that growth substance production by zones of the coleoptile below the tip cannot take place until a low concentration is reached.

The growth of *Avena* coleoptiles after decapitation has been previously studied by Dolk (1926) and by Heyn (1931). These investigators found exactly the same type of response as in the present case, but since they both worked at lower temperatures (21° C. and 23° C. respectively) they found the minimum to occur somewhat later. As mentioned in Part I, the appearance of new growth after the minimum is due to the production of growth substance by the uppermost region of the coleoptile stump, which previously did not produce growth substance. The growth of a decapitated coleoptile before the minimum, represents, then, the growth caused by whatever growth substance was left in the plant after removal of the tip.

Rate of Growth of Plants supplied with Low Concentrations of Growth Substance.

In the next series of experiments, plants were supplied with agar blocks containing 9·9 and then 14·5 "plant units" of growth substance. Now, the rate of passage of growth substance from block to plant depends upon the concentration of growth substance in the block. Hence, even by allowing the block to remain for a very long time upon a coleoptile, the complete transference of the growth substance to the plant would be impossible. It was found much more convenient to remove the block after a definite time and to determine, as in the previous section, the amount of growth substance which had actually entered the plant. For comparison with plain decapitated coleoptiles, the blocks were allowed to remain 105 minutes, that is, until physiological regeneration would have occurred if the plants had not been supplied with growth substance.

The mean growth rates of these plants are shown in fig. 2, in which the curve for decapitated coleoptiles without growth substance is inserted for comparison. The data are included in Table III. The 9·9 unit curve is somewhat irregular, but falls, in general, although more slowly than that of plain de-

capitated coleoptiles, to a rather low value after 3 hours without exhibiting any minimum. The 14·5 unit curve, is, until removal of the block, nearly constant at a value slightly higher than that of the initial growth rate of plain decapitated coleoptiles. After removal of the block the rate falls through a minimum and rises to a constant value. The minimum is not so low and is reached one half-hour earlier than the minimum of plain decapitated coleoptiles. The shape of the curve resembles greatly, however, that of fig. 1.

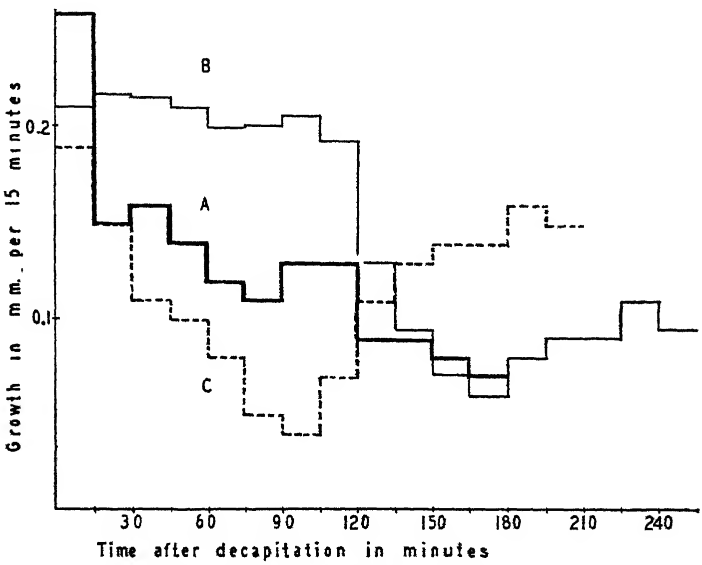

FIG. 2.—Growth rates of decapitated coleoptiles supplied with small quantities of growth substance. Curve A, 9·9 plant units of growth substance; curve B, 14·5 plant units of growth substance; curve C, no growth substance.

The coleoptile recommences the production of growth substance in this case, when it has reached the minimum growth rate, which is after 3 hours. The total growth between decapitation and this minimum is due (*a*) to the *added* growth substance (*i.e.*, given up by the block), (*b*) to the *residual* growth substance left in the plant after decapitation. It is clear that the growth caused only by the added growth substance is the total growth from the time of decapitation to the minimum, less the amount of growth of plain decapitated coleoptiles to their minimum. If it be assumed, as seems justified, that at the end of 3 hours the 9·9 unit curve also begins to mount toward a new constant level, the growth due to the 9·9 unit block may be determined in the same way.

Growth Rates of Plants supplied with Higher Concentrations of Growth Substance.

The rates of growth due to successively higher concentrations of growth substance was next studied, and Table III gives the means of the determinations at each concentration. The growth rates at four of the concentrations are plotted in fig. 3. In each case, except one at very high concentration, the addition of more growth substance to the plant results in an increase of growth. In general, also, the response to a large concentration of growth substance is immediate, that is, even in the first period the growth is larger than that of plain decapitated coleoptiles. After removal of the block the curves drop

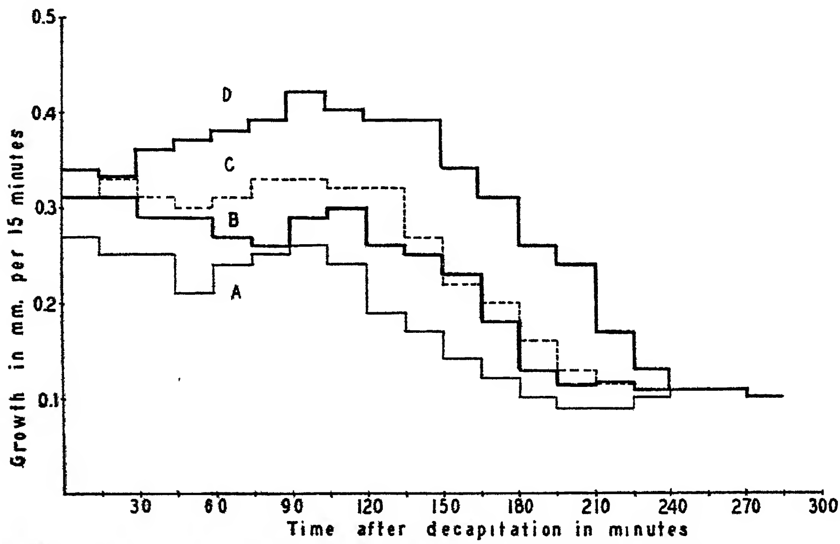

Fig. 3.—Growth of decapitated coleoptiles supplied with large quantities of growth substance. Curve A, 19·3 plant units of growth substance; curve B, 29·0 plant units of growth substance; curve C, 39·5 plant units of growth substance; curve D, 116·0 plant units of growth substance.

to a low constant value, which is approximately the same in each case. The time between the removal of the block and the attainment of this low value is, however, longer the higher the concentration. The fact that this final constant level is lower for plants supplied with growth substance than for the plants of fig. 1 may be attributed to the greater utilization of other necessities of growth in the presence of more growth substance.

None of the curves of fig. 3 exhibit a clear minimum. Heyn has stated that the removal of the block is exactly similar to removal of the original tip, and that the production of new growth substance commences at a definite time

later. If this be true, no minima would be expected with the higher concentrations of growth substance, since the growth substance left after the removal of the block would give rise to a rather large amount of growth. This growth would obscure the minima. The quantitative relation between growth and growth substance which may be obtained from the present data with the aid of a different assumption, however, lead us to believe that Heyn's interpretation is not the correct one, and that the true reasons for the absence minima are those which will be brought forward below.

The Linear Relationship between Growth and Growth Substance.

The zones which produce growth substance after physiological regeneration of a tip in a decapitated coleoptile do not, in the presence of the tip, produce any appreciable amount. This suggests that the growth substance has, as a secondary function, the inhibition, by its presence in sufficiently high concentrations, of the production of growth substance by the lower zones. In a plain decapitated coleoptile or in one supplied with a small amount of growth substance, removal of the tip, or of the agar block, is followed by transport of all of the small amount of residual growth substance from the top zones to the lower (Van der Wey, 1932). The result is that when the concentration of growth substance in the top of the stump has become low enough to permit regeneration, the growth rate of the coleoptile as a whole has become very small, and the growth curve therefore passes through a minimum. If, however, a decapitated coleoptile is supplied with a large amount of growth substance, a different result would be expected. After removal of the block, the growth substance which is left in the upper zones is transported to the lower, but these zones are unable to use such a large amount as quickly as it is brought in. The result is that while the concentration in the uppermost zone falls to a value sufficiently low to permit regeneration, the concentration in the lower zones remains high, they grow continuously, and no minimum in the total growth rate is apparent. The essential difference between the situation of plants supplied with large and with small amounts of growth substance is, then, one of distribution.

In accordance with this view there is, for plants supplied with large concentrations of growth substance, no exact time at which growth due to the growth substance from the block ceases, and that due to growth substance from the regenerated tip commences. As an approximation it can be assumed that the time at which the growth reaches its final low constant level is the

time at which the growth substance from the block is used up. The correction is in any event small.

The total growth of these plants from time of decapitation to establishment of the final level, less that due to the residual growth substance, represents, then, in each case the growth caused by the amount of growth substance which has entered the plant, and this amount is, as already pointed out, proportional to the concentration applied.

Table IV.—Total Growth and Additional Growth Resulting from Application of Growth Substance for 105 minutes.

Units applied.	Number of plants.	Total growth (see text).	Time (see text).	Additional growth (see text).	$\frac{\text{Growth}}{\text{Units applied.}}$
		mm.	min.	mm.	
0	6	0·71	105	0	—
9·9	3	1·54	180	0·83	0·084
14·5	10	2·03	180	1·32	0·091
19·3	7	2·57	180	1·86	0·096
29·0	7	3·37	195	2·66	0·092
39·5	6	3·91	217	3·20	0·081
43·5	7	4·05	217	3·34	0·076
58·0	6	4·98	225	4·27	0·074
79·0	4	4·96	225	4·25	0·054
116·0	6	5·22	240	4·51	0·039

Table IV gives the growth thus obtained from each concentration of growth substance. From the total growth, from decapitation to the final level, was subtracted the total growth, to their minimum, of the decapitated coleoptiles of fig. 1. For comparison of the growth per unit of growth substance, this value is in each case also given. For the four lowest concentrations this ratio is, within a few per cent., constant. Above 29 units the ratio decreases somewhat, that is, a further addition of growth substance produces proportionately less growth. Above 58 units the ratio decreases sharply; in fact, 116 units gives but little more growth. Fig. 4 summarizes the amounts of growth from Table IV, plotted against growth substance concentration, and shows that the relationship of growth response of coleoptiles to growth substance is, up to 29 units, linear, while above 58 units more growth substance, on the average, produces little increase in growth. For comparison the curvatures produced by unilateral growth are also plotted against concentration of growth substance (dotted line). The limiting concentration can be seen to be much higher for straight growth than for curved growth, owing probably to elastic and other counteracting forces in the latter. A calculation of the

growth necessary to produce a given curvature shows that for this a much smaller amount of new tissue is formed, per unit of growth substance, than in straight growth.

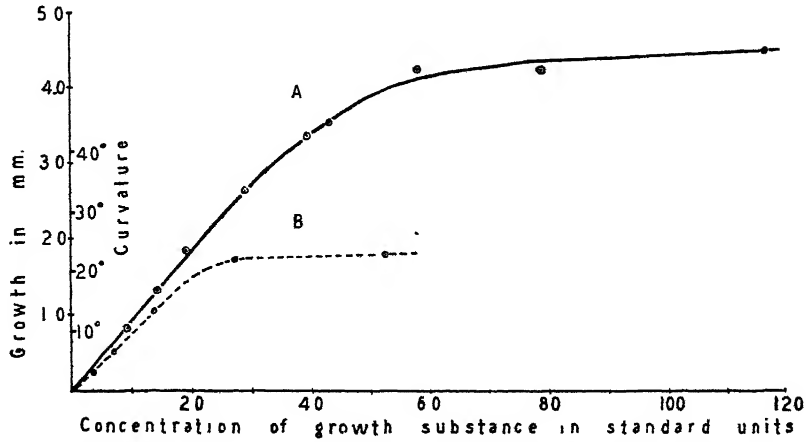

Fig. 4.—Amount of growth resulting from different quantities of growth substance. Curve A, vertical growth; curve B, curved growth.

5. The Composition of the Cell Wall.

In order to convert growth, in terms of coleoptile length, into amounts of material actually laid down, the relation between coleoptile length and amount of cell-wall material must be known.

The material used in these experiments consisted of coleoptile cylinders, with tips removed, either of young plants of the size used in the previous work, or of old plants from which the first leaves would soon break. It was found that although the weight of "cell walls" per millimetre was greater in the older plants, yet the composition of the cell wall was the same. The old plants were more convenient to use, since they provided much more material with which to work.

Table V gives the dry weights per millimetre of a number of samples of young coleoptiles. The weights depend considerably upon the conditions of drying, since although samples dried at the same time (100° for 24 hours) agree fairly well, there is more variation between samples dried at different times. Nevertheless, the dry weight per unit length is relatively constant. This constancy of dry weight per millimetre might have been expected, since the coleoptile is, except for its tip, of very nearly uniform diameter, which varies but little from plant to plant.

Table V.—Dry Weights of Coleoptiles.

Aggregate length of coleoptiles in mm.	Weight in mg.	Weight per mm., grams . 10^{-5}.
332	16·9	5·09
348	18·0	5·17
341	18·6	5·46
349	19·2	5·51
408	25·8	6·32
409	26·0	6·35
207	12·3	5·95
132	8·3	6·30
173	10·5	6·08
179	11·6	6·50
336	17·6	5·25
389	17·0	4·25
643	33·9	5·23
200	10·7	5·35
306	17·8	5·81
300	17·1	5·70
		Mean 5·81

The next step was to obtain cell walls free of cytoplasm and cell sap. In order to accomplish this, the samples were thoroughly ground, and extracted several times with cold water. This was followed by 2 hours of extraction with repeated portions of hot water. The residue was retained upon a coarse filter, through which the colloidal solution obtained from the washings readily

Table VI.—Weight of Cell Walls from Young Coleoptiles.

Aggregate length of coleoptiles in mm.	Weight of cell wall in mg.	Weight of cell wall per mm. in grams . 10^{-5}.
498	8·1	1·63
635	11·4	1·79
687	10·9	1·50
745	12·8	1·72
731	11·3	1·55
637	10·5	1·65
		Mean 1·64

This fraction, which is not dissolved or suspended in water, can be considered as the cell wall. It was dried to constant weight at 95° C. Table VI gives the amounts of this fraction obtained from several samples of young coleoptiles.

Extraction of the cell-wall fraction for several hours with ether gave no decrease in dry weight, and it was therefore concluded that no appreciable amount of fatty substances were present. The pectic substances were extracted by the method of Nanji and Norman (1928), in which the cell-wall samples are heated at 80° C. for 24 hours with 1/2 per cent. ammonium oxalate. Results for two such extractions are included in Table VII.

Table VII.—Analysis of Cell Walls of Coleoptiles.

Weight of cell walls.	Weight after extraction with oxalate.	Weight after extraction with 2 per cent. H_2SO_4.	Weight after extraction with 2 per cent. NaOH.	Total N.	Protein.
22·3	20·4	11·4	8·6	—	—
47·6	43·9	25·0	17·4	—	—
21·0	—	—	9·7*	—	—
25·2	—	—	11·1*	—	—
22·8	—	—	10·3*	—	—
—	14·4	—	—	0·322	1·93
—	19·5	—	—	0·392	2·08

* These three determinations were made after previous extraction with oxalate and with 2 per cent. H_2SO_4.

The amount of cellulose present in material of this kind may be determined approximately by the "crude fibre." The samples were heated successively with 2 per cent. H_2SO_4, 2 per cent. KOH, and with alcohol, and the residue dried and weighed. Pentosans may remain to a small extent as impurities, as has been shown by Lüdtke (1931). For unlignified tissues, however, the crude fibre is principally cellulose. The degradation was also carried out in steps upon young and old coleoptiles, in order to compare the composition of the two. These results are included in Table VII. It may be seen that the amount of substance removed at each step does not depend upon the age of the coleoptile. Since this is so, the ratio of the water-insoluble fraction to the crude fibre was determined several times upon old coleoptiles (see Table VII).

Determinations of the nitrogen in the samples were carried out by the micro-Kjeldahl method. An approximation to the per cent. of protein present in the wall may be obtained by multiplying the percentage of nitrogen by 6·2. The nitrogen found in two samples of cell walls, together with the calculated protein, is included in Table VII.

A summary of the results is given in Table VIII, in which the different fractions of the cell wall are expressed as percentages of the total average dry weight. It might seem at first sight rather surprising that only about 12 per

Table VIII.—Summary of Composition of Coleoptile Cell Wall.

Fraction.	Percentage of cell wall.	Percentage of total coleoptile.
Removed by oxalate (pectic substances)	8	2·3
Removed by dilute acid less protein (hemicellulose)	28	8·2
Removed by dilute alkali (further pentosans)	10	2·8
Protein	12	3·1
Residue (cellulose)	42	11·9
	100	28·3

cent. of the coleoptile may be considered as consisting of cellulose, and that such large amounts of hemicelluloses and proteins are present in the cell wall. Link (1929) has analysed *Zea* seedlings, of an age comparable to that of the Avena plants used here, and found that only 14–18 per cent. of their total dry weight is pure cellulose, and that 10–12 per cent. is pure xylan.

It will be assumed that the increment of length produced by the action of growth substance is composed of normal tissue, that is, that the processes of formation of new material keep up with the processes of elongation, irrespective of whether these two sets of processes are connected or not. That this is actually so is indicated by the following experiment: coleoptiles were supplied every 2 hours for 14 hours with fresh agar blocks containing 100 units of growth substance. At the end of this time the coleoptiles were cut off, dried, and weighed, together with normal control coleoptiles. The results were as follows: the plants supplied with growth substance increased in length an average of 1·9 cm. (12 plants) while normal coleoptiles in the same time increased by 1·0 cm. (6 plants). The dry weight of the coleoptiles supplied with growth substance was $5 \cdot 2 \,.\, 10^{-2}$ mg. per millimetre, while that of normal coleoptiles carried out at the same time was, in two samples, 5·1 and $5 \cdot 2 \,.\, 10^{-2}$ mg. per millimetre.

6. The Volume of the Cell Walls.

A determination was made of the volume of new cell-walls which appear as a result of the action of a known amount of growth substance. Since the estimation of the area of the cell-wall is at best rather uncertain, the coleoptile can for the present purposes be considered as a hollow cylinder. This simplifies the calculations without greatly affecting the result. For longitudinal growth, only the longitudinal walls need be considered.

The inside and outside diameters, the diameters of the individual cells and hence the diameters of each cell layer were measured in a large number of

coleoptiles. Since the average number of cell layers was 6, the total area of tangential walls could be calculated, and from the number of cells in each layer the area of radial walls was also obtained. The total area of cell wall per millimetre of coleoptile was thus found to be $40 \cdot 3\ mm^2$. The average thickness of the cell walls was found to be $0 \cdot 4 \mu$.

7. Ratio of Cell Wall Constituents produced to Growth Substance Used.

From fig. 4, it is clear that the response of the coleoptile bears a linear relation to the quantity of growth substance if the latter is smaller than about 35 units. We will therefore consider the response to 29·0 units, which is well within this range.

Table IV shows that the addition of 29 units produces a growth of 2·66 mm. From analysis of the coleoptile, Table VI, it was found that the weight of cell walls in 1 mm. is $1 \cdot 64 . 10^{-2}$ mg. We will consider for the moment that only the actual cellulose of the cell wall has been laid down by the action of growth substance, if such a reaction, in fact, takes place. This assumption is to some extent justified by the consideration that the principal skeletal material of the coleoptile is certainly the crystallized cellulose. It is clear that this procedure will lead to a minimum value for the carbohydrate deposited as a result of growth substance action. Table VIII has shown that the cellulose (crude fibre) constitutes 42 per cent. of the cell wall, and hence the amount of cellulose deposited as a result of 29 units is

$$2 \cdot 66 \times 1 \cdot 64 . 10^{-2} \times 0 \cdot 42 = 1 \cdot 83 . 10^{-2} \text{ mg.}$$

Now cellulose is made up of glucose residues ($C_6H_{10}O_5$), of molecular weight 162, and 1 millimole contains $6 \cdot 06 . 10^{20}$ such residues. Therefore, the number of such glucose residues laid down as cellulose under the action of 29 units is

$$1 \cdot 83 . 10^{-2} \times \frac{1}{162} \times 6 \cdot 06 . 10^{20} = 6 \cdot 84 . 10^{16} \text{ residues.}$$

In order to arrive at a figure for the amount of growth substance involved, we shall use the value given by Kögl and Haagen-Smit (1931), since, while their substance is not necessarily identical with that used here, it is closely similar in action, and was obtained by them in the crystalline state. According to them, one unit is equal to 1/30,000,000 mg. The unit which they use is defined as giving a 10° curvature, whereas ours is defined as giving a curvature

of 1°. The blocks which they use have a volume of 2 mm.³ and hence by the procedure of Table IV of Thimann and Bonner (1932), 58 per cent. of the growth substance passes out of the block during their test. The amount passing out of the block in our test is 15 per cent. and hence we need to have four times their amount in our block to give the same curvature. Our unit is then

$$4 \times 3\cdot3 \,.\, 10^{-8} \times 1/10 = 1\cdot3 \,.\, 10^{-8} \text{ mg.}$$

In this experiment $29 \times 1\cdot3 \,.\, 10^{-8} = 3\cdot8 \,.\, 10^{-7}$ mg. were used.

Table I shows that when blocks are placed flat upon coleoptiles, as in this experiment, 34 per cent. of the growth substance present in the block passes into the plant, that is, in this case,

$$0\cdot34 \times 3\cdot8 \,.\, 10^{-7} = 1\cdot3 \,.\, 10^{-7} \text{ mg.}$$

pass into the plant. The mean molecular weight of the growth substance obtained by Kögl and Haagen-Smit is 341, and this figure is checked by the value obtained in this laboratory. The number of molecules of growth substance entering the plant is then

$$6\cdot06 \,.\, 10^{20} \times 1\cdot3 \,.\, 10^{-7} \times 1/341 = 2\cdot31 \,.\, 10^{11} \text{ molecules.}$$

We have, then, from above, $2\cdot31 \,.\, 10^{11}$ molecules of growth substance for $6\cdot8 \,.\, 10^{16}$ molecules of $C_6H_{10}O_5$, that is, one molecule of growth substance has acted in the laying down of $3\cdot0 \,.\, 10^5$ $C_6H_{10}O_5$ residues. Since this ratio is greatly different from 1, it is at once clear that *the growth substance is not involved directly in the deposition either of cellulose, or of total cell-wall carbohydrate.*

We will now compare the number of molecules of growth substance with the number of micelles of cellulose deposited. As Katz (1928) points out, the micelles of fibres, since they are not visible even with ultra-violet light, must be shorter than 500 A. Katz gives the most probable size of the micelles as 60–110 A. The unit cell of cellulose is $8\cdot7 \times 7\cdot9 \times 10\cdot3$ A, and contains four glucose residues (Meyer and Mark, 1928). If we take the micelles as $87 \times 40 \times 103$ A., a figure which accords with that of Katz, they each contain 2000 glucose residues. Since 1 growth substance molecule acts in the deposition of $3 \,.\, 10^5$ glucose residues, it acts, therefore, in the deposition of

$$3\cdot10^5 \times 1/2000 \text{ or } 140 \text{ micelles.}$$

Since there is no reason to believe that the micelles of cellulose in young tissues, such as coleoptiles, are any larger than those of the cotton and ramie fibres,

with which the micelle size determinations were made, it is clear that *there cannot be any one to one correspondence between growth substance molecules and the micelles formed in growth.*

A second, less reliable, method for determining the number of molecules of cellulose laid down by one molecule of growth substance is as follows.

It was found in Section 6 that the total area of longitudinal cell walls per millimetre of coleoptile length was $40 \cdot 3$ mm.^{2} and the mean wall thickness was $0 \cdot 4\ \mu$. Hence the volume of the cell-walls is

$$40 \cdot 3 \times 4 \cdot 10^{-4} = 1 \cdot 61 \,.\, 10^{-2} \text{ mm.}^3.$$

As above, the unit of cellulose structure is the block containing four glucose residues, and having dimensions $8 \cdot 7 \times 7 \cdot 9 \times 10 \cdot 3$ A., or volume $7 \cdot 08 \,.\, 10^{-19}$ mm.^{3}. The specific gravity of cellulose will not be greatly different from that of the other cell-wall material, and we can assume the cell-wall material to consist, therefore, of 42 per cent. cellulose by volume. Hence 1 mm. of coleoptile contains

$$1 \cdot 6 \,.\, 10^{-2} \times 1/7 \cdot 08 \,.\, 10^{-19} \times 0 \cdot 42 = 0 \cdot 96 \,.\, 10^{16} \text{ unit blocks.}$$

Since each block contains four hexose residues, there are $3 \cdot 82 \,.\, 10^{16}$ hexose residues per millimetre of coleoptile.

An increase in length of $2 \cdot 66$ mm., the result of the application of 29 units of growth substance, therefore involves the production of $3 \cdot 82 \,.\, 10^{16} \times 2 \cdot 66$ or $10 \cdot 2 \,.\, 10^{16}$ new hexose residues. Since no allowance has been made for the volume taken up by water in the wall, this figure may be somewhat too high. Considering the approximate nature of the determination of the cell-wall volume, the agreement between this figure and that obtained above from the weight of the wall must be regarded as satisfactory. Using the above figure, we find that one molecule of growth substance leads to the production of $4 \cdot 6 \,.\, 10^5$ hexose residues, instead of $3 \,.\, 10^5$ as in the first calculation.

Since from the above it is scarcely possible to distribute a stoichiometrical relationship between the growth substance and the materials of the cell wall, another possibility was investigated, namely, that the growth substance is distributed in a monomolecular layer over the surface of the cell, and is thus by some means able to promote the elongation of the wall area which it covers.

According to the analyses of Kögl and Haagen-Smit, the growth substance has the approximate formula $C_{20}H_{36}O_4$. Since it is also known to be an acid (Dolk and Thimann, 1932) one may apply Langmuir's measurements on surface films of the fatty acids, from which a C_{20} acid will have a length of $27 \cdot 8$ A.,

and width 4·7 A. The molecule thus has a maximum area of $1 \cdot 3 \, . \, 10^{-12}$ mm.^{2}. As stated above, the cell wall has an area of 40·3 mm.^{2} per millimetre of coleoptile, and hence 2·66 mm. have an area of 107 mm.^{2}. The number of molecules of growth substance involved is $2 \cdot 3 \, . \, 10^{11}$, which, from the above value, can only cover a maximum area of 0·3 mm.^{2}, and if orientated at the surface, can occupy even less. Therefore, to form a monomolecular layer only on the surface of the new cell wall laid down, each molecule of growth substance would have to cover 357 times its own maximum area. Now for the growth substance to act by producing any change in permeability, it must, presumably, produce a change in surface activity; to do this it must form at least a monomolecular layer upon the cell wall, which is clearly impossible. Furthermore the measurements of Rideal (1925) and of Langmuir (1927) show that the rate of passage of water from a liquid surface into the vapour is greatly decreased by the presence of surface films of fatty acids. Thus it is likely that any effect of growth substance upon permeability to water would be in the direction of decreasing rather than increasing it. *The theory that the action of growth substance is one of permeability change thus appears extremely improbable.*

We therefore conclude that the growth substance does not act in any direct manner in producing cell elongation. There are a number of possible mechanisms by which indirect action can occur; the data do not at present permit of selection between these, but an attempt is being made to obtain further evidence.

Summary.

(1) The mechanism of the action of growth substance in promoting cell elongation in Avena coleoptiles has been studied.

(2) The amount of growth resulting from the application of a given amount of growth substance to decapitated coleoptiles was measured by a kinematographic method. A linear relationship between growth substance added and growth produced was found to hold over a considerable range.

(3) The fraction of the applied growth substance which enters the plant under the particular conditions adopted was determined by the curvature technique.

(4) The composition and the volume of the cell wall per unit length of coleoptile were determined.

(5) From these data the relation between the growth substance entering the plant and the cell-wall material produced as a result of its action was calculated.

(6) The calculations make clear that:

(*a*) The growth substance does not play any stoichiometrical part in the deposition either of cellulose or of the total cell wall.

(*b*) The growth substance does not act by producing a monomolecular layer upon the new cell wall laid down, and therefore very probably does not affect growth through changes in permeability.

The growth substance therefore acts in some indirect way whereby each molecule exerts its influence a number of times.

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577 . 158 . 17

The Chemical Nature of the Active Group in the Enzyme Glucose Dehydrogenase.

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It has been shown (Harrison, 1931) that an enzyme, glucose dehydrogenase, which brings about the oxidation of glucose, can be extracted from the liver of various animals. The product of this oxidation of glucose has been shown to be *d*-gluconic acid (Harrison, 1932). It seemed likely that during this conversion of glucose to gluconic acid, the ring structure of glucose would be broken, with the intermediate formation of the open chain form of glucose containing a free aldehyde group. This free aldehyde group of glucose might then be capable of oxidation by another enzyme, the Schardinger enzyme, which appears to bring about the oxidation of any soluble aldehyde. The Schardinger enzyme is widely distributed in the body tissues, and it seemed possible that it might collaborate in this way with glucose dehydrogenase in the oxidation of glucose in the body. On carrying out an experiment to test this, however, it was surprising to find that the addition of the Schardinger enzyme to glucose and glucose dehydrogenase, so far from accelerating the oxidation, brought about a complete, or almost complete, inhibition of the oxidation of glucose by the dehydrogenase. Further experiments described below showed that the inhibition was due to the Schardinger enzyme itself and not to some impurity in the enzyme preparation.

It was decided to investigate this inhibition further, for it is well known that the Schardinger (or xanthine) oxidase induces the oxidation of only aldehydes and certain purines. If it could be shown that the inhibition was due to an oxidative destruction of the glucose dehydrogenase by the Schardinger enzyme, it might well throw light on the chemical structure of the dehydrogenase enzyme.

Experimental.

In measuring the rate of oxidation of glucose, the well-known Thunberg technique was employed, in which the rate of decolorization of methylene blue by glucose and the enzyme is measured. The reaction is carried out in

evacuated tubes, the methylene blue acting as the hydrogen acceptor and becoming reduced to the colourless leuco-compound during the oxidation of glucose. In the preliminary experiments, the glucose dehydrogenase was prepared from ox liver by the method previously described (Harrison, 1931). It was shown later by Mann (1932, *a*) that an activator is concerned in the oxidation of glucose by the dehydrogenase. The above preparation contained both the dehydrogenase and the activator or co-enzyme. In most of the experiments described in this paper, the dehydrogenase and its co-enzyme were extracted from acetone ox liver and separated and purified by the method recently described (Harrison, 1933). The Schardinger enzyme or xanthine oxidase was the ether-extracted "whey" preparation made from milk as described by Dixon and Kodama (1926). The question as to whether the Schardinger and the xanthine oxidase are one and the same enzyme or two distinct enzymes has not apparently been definitely settled, but the preparation used was capable of oxidizing both aldehydes and hypoxanthine and, to avoid confusion, will be referred to subsequently in this paper as the xanthine oxidase preparation.

The enzyme solutions were prepared by dissolving a suitable amount of the solid enzyme preparation in water, bringing to the required p_H by cautious addition of NaOH and centrifuging to obtain a perfectly clear solution. Unless otherwise stated, the amount of glucose dehydrogenase and co-enzyme used in each Thunberg tube was equivalent to 0·8 gm. acetone liver, this amount usually giving a convenient time of reduction of methylene blue. In all experiments except the first, 0·25 c.c. of a 1/5000 aqueous solution of methylene blue was added to each tube. The glucose used was a 2M. solution of pure dextrose. The total volume of the solution in each Thunberg tube was always 1·55 c.c. unless otherwise stated, this volume being made up by addition of the requisite volume of distilled water. The tubes containing the solutions were evacuated on the water pump, filled with nitrogen, re-evacuated and immersed in a water bath at 37° C.

Table I shows the complete inhibition produced by strong xanthine oxidase on the oxidation of glucose in presence of glucose dehydrogenase and its co-enzyme. The p_H of the solutions was 7·2 (0·5 c.c. methylene blue in a total volume of 2 c.c. was used in this experiment).

It should be noted that a high figure for the time of decolorization of methylene blue implies a low enzyme activity and *vice versa*.

The activity of this xanthine oxidase was such that the time of reduction with 0·4 c.c. xanthine oxidase and 0·2 mg. hypoxanthine was 2 minutes, while

Table I.

Glucose dehydrogenase with co-enzyme.	2M. glucose.	Xanthine oxidase.	Time in minutes for decolorization of methylene blue.
c.c.	c.c.	c.c.	
1	—	—	> 180
1	—	0·4	180
1	0·1	—	12½
1	0·1	0·4	180

in presence of 1 c.c. of dehydrogenase + co-enzyme solution, the time was 1½ minutes, showing that while xanthine oxidase inhibits glucose dehydrogenase, glucose dehydrogenase does not inhibit the activity of xanthine oxidase towards hypoxanthine. The activity of the xanthine oxidase toward acetaldehyde was likewise unimpaired in the presence of the dehydrogenase + co-enzyme solution. The inhibition cannot, therefore, be due to a mutual precipitation or inactivation of the two enzymes. Rennin had been used in making the whey preparation of xanthine oxidase, but control experiments with glucose dehydrogenase and rennin showed that the latter produces no inhibition in the activity of the dehydrogenase.

It seemed likely that the question as to whether the inhibiting action of xanthine oxidase preparations was due to the oxidase itself could be settled by repeating the experiments in presence of uric acid. Uric acid is known to be a specific inhibitor towards the xanthine oxidase (Dixon and Thurlow, 1924), and should therefore diminish or abolish the inhibiting action of the oxidase on glucose dehydrogenase. Unfortunately, it was found that uric acid by itself in sufficiently high concentration interferes with the oxidation of glucose by the dehydrogenase, and it could not therefore be used to settle this point.

It was found that the inhibiting action of xanthine oxidase towards glucose dehydrogenase disappears if the xanthine oxidase be first heated. Table II shows the effect of previously heating xanthine oxidase for 5 minutes at progressively increasing temperatures. The p_H of the solutions was 7·5. After heating, the activity of the xanthine oxidase towards hypoxanthine at 37° C. is shown in the last column, the corresponding inhibiting action on glucose dehydrogenase being shown in the preceding column. (In this experiment, in testing the activity of the oxidase towards hypoxanthine, 0·5 c.c. xanthine oxidase, 0·16 mg. hypoxanthine and 0·25 c.c. methylene blue were used in a total volume of 1·3 c.c.)

It can be seen that after heating xanthine oxidase for 5 minutes at tempera-

Table II.

No.	Dehydrogenase + co-enzyme.	2M. glucose.	Xanthine oxidase.	Time in minutes for reduction of methylene blue with glucose.	Methylene blue reduction time with hypoxanthine.
	c.c.	c.c.			
1	1	0·1	None	16	—
2	1	0·1	0·4 c.c. (unheated) .	> 150	$2\frac{1}{2}$
3	1	0·1	0·4 c.c. (heated to 55°)	> 150	$3\frac{3}{4}$
4	1	0·1	0·4 c.c. (heated to 60°)	> 150	$5\frac{1}{4}$
5	1	0·1	0·4 c.c. (heated to 65°)	> 150	7
6	1	0·1	0·4 c.c. (heated to 70°)	29	23

tures from 55° C. to 65° C. it still has the power to oxidize hypoxanthine actively and it still produces a large inhibition on the oxidation of glucose by glucose dehydrogenase. After heating at 70° C., however, the greater part of the activity of the oxidase towards hypoxanthine has disappeared, and at the same time, its power to inhibit the oxidation of glucose has been largely destroyed. This affords strong evidence that the inhibiting action of xanthine oxidase preparations is caused by the xanthine oxidase itself and not by some other inhibiting substance present in the solutions.

With weak solutions of xanthine oxidase, the degree of inhibition appears to be roughly proportional to the amount of oxidase added. The reaction is not instantaneous, and with weak oxidase solutions the amount of inhibition is much increased by previously incubating the two enzymes together.

It was now desirable to ascertain whether the inhibition was due to the xanthine oxidase causing an actual destruction of the glucose dehydrogenase or merely interfering with the reaction. The obvious method would have been to add xanthine oxidase to glucose dehydrogenase and then to remove it before adding glucose to test whether a permanent destruction of the dehydrogenase had occurred. Unfortunately, both enzymes are precipitated by half saturated ammonium sulphate and a method for quantitative separation could not be found. It became possible to test the point, however, owing to the observation that the inhibiting action of xanthine oxidase is very largely prevented by the presence of a sufficient concentration of phosphate. The experiments hitherto described were carried out in aqueous solution in the absence of added buffer solution. As previously pointed out (Harrison, 1931, 1932), the protein in the dehydrogenase solution possesses ample buffering power. Frequent tests of the p_H of the solutions at the end of the experiment (by adding indicators through the side tube of the Thunberg tube after the

methylene blue had been decolorized) confirmed this. The effect of phosphate buffer solution is shown by the following typical experiment in which the experimental conditions were the same as those given in Table II. The time of reduction of methylene blue by glucose with the dehydrogenase + co-enzyme solution alone was 17 minutes, while in presence of xanthine oxidase the time was 70 minutes with M/100 phosphate buffer (p_H 7·5) and 19 minutes with M/25 phosphate buffer. Increasing the phosphate concentration four times has thus almost completely removed the inhibiting action of xanthine oxidase on glucose dehydrogenase. A number of control experiments showed that phosphate buffer in concentrations up to M/15 did not affect the activity of either of the enzymes themselves; that is, the velocity of oxidation of glucose by the dehydrogenase-co-enzyme mixture and that of acetaldehyde or hypoxanthine by xanthine oxidase were unaffected by concentrations of phosphate more than sufficient to abolish the inhibition by xanthine oxidase on the dehydrogenase.

This property of phosphate of preventing the xanthine oxidase inhibition made it possible to determine whether the oxidase actually destroys the dehydrogenase or whether it merely interferes with the oxidation of glucose by the dehydrogenase.* If the two enzymes be first incubated together, then the subsequent addition of phosphate along with the glucose and methylene blue should not prevent the xanthine oxidase inhibition if the xanthine oxidase has already brought about a destruction of the dehydrogenase. If, on the other hand, xanthine oxidase inhibits by interfering with the actual oxidation of glucose, this inhibition should be removed by phosphate in spite of the previous incubation of the two enzymes together.

Table III shows the result of such an experiment. Six tubes each containing 0·9 c.c. of dehydrogenase + co-enzyme solution (p_H 7·6) were incubated aerobically at 37° C. for 15 minutes, tubes 5 and 6 containing in addition 0·1 c.c. xanthine oxidase solution (p_H 7·6) (equivalent to 2·5 mg. of the dry preparation). After incubation, 0·1 c.c. of 2M. glucose and 0·25 c.c. methylene blue were added to each tube, and 0·1 c.c. xanthine oxidase previously incubated alone in the same way was added to tubes 3 and 4. Phosphate buffer p_H 7·6 was added to a final concentration of M/15 to control tube No. 2 and to tubes 4 and 6, while an equal volume of water was added to control tube No. 1 and to tubes 3 and 5. The total volume in each tube was made up to 1·55 c.c. with distilled water and the tubes were evacuated and immersed in the water bath at 37° C.

* I am indebted to Professor E. Mellanby for this suggestion.

Table III.

No.	—	Substance added after incubation.	Time in minutes for decolorization of methylene blue.
1	Dehydrogenase + co-enzyme incubated alone	Water	19
2	,, ,,	Phosphate	18
3	,, ,,	Xanthine oxidase + water	98
4	,, ,,	Xanthine oxidase + phosphate	24
5	Dehydrogenase + co-enzyme incubated with xanthine oxidase	Water	>100
6	,, ,,	Phosphate	72

Comparing the reduction times in tubes 4 and 6, it is evident that the inhibition by xanthine oxidase still takes place even in presence of phosphate if the xanthine oxidase and the dehydrogenase have previously been allowed to react together before the addition of the phosphate. This shows, therefore, that the inhibiting action of xanthine oxidase is due to an actual destruction of the glucose-oxidizing enzyme. The fact that the reduction time in tube 6 is shorter than in tube 5 is doubtless owing to the fact that in the latter tube the destructive action of the oxidase on the dehydrogenase occurs not only during the preliminary 15 minutes incubation but is allowed to go on during the much longer subsequent reaction period, while in tube 6, the addition of phosphate after the preliminary incubation prevents any such further destruction of the dehydrogenase. This experiment was then repeated with the dehydrogenase without co-enzyme, the co-enzyme solution being added together with the glucose after the incubation period. Exactly similar results were obtained. This shows that the destructive action of xanthine oxidase is on glucose dehydrogenase itself and not on the co-enzyme.

Experiments were now carried out to determine whether the destruction of glucose dehydrogenase by xanthine oxidase is due to an oxidation of the former enzyme by the latter. If this were so, incubation of the two enzymes in the absence of oxygen or other hydrogen acceptor should prevent the destruction of the dehydrogenase. Table IV shows that this actually happens. A relatively dilute solution of xanthine oxidase was used in this experiment, such that only a small inhibition was produced without the preliminary incubation period. In this way, any differences between a destructive aerobic incubation and a non-destructive anaerobic incubation would be

Table IV.

No.	—	Time in minutes for decolorization of methylene blue.
1	Dehydrogenase incubated with O_2	13½
2	Dehydrogenase incubated with O_2, xanthine oxidase then added	26
3	Dehydrogenase + xanthine oxidase incubated with O_2	36
4	Dehydrogenase incubated without O_2	13
5	Dehydrogenase + xanthine oxidase incubated without O_2	29
6	Dehydrogenase + methylene blue incubated without O_2	13
7	Dehydrogenase + methylene blue + xanthine oxidase incubated without O_2	38

well brought out. Vacuum tubes Nos. 1 to 7 each contained 0·8 c.c. glucose dehydrogenase (p_H 7·4). Tubes Nos. 3, 5 and 7 received in addition 0·1 c.c. dilute xanthine oxidase (p_H 7·4) while tubes 6 and 7 also contained 0·25 c.c. methylene blue to act as an alternative hydrogen acceptor in the absence of oxygen. Tubes 4, 5, 6, 7 were then evacuated and incubated at 37° C. for 30 minutes, while tubes 1, 2 and 3 were incubated under the same conditions in the presence of air. The tubes were then opened, 0·1 c.c. of xanthine oxidase (separately incubated for 30 minutes) was added to tube No. 2, 0·2 c.c. 2M. glucose and 0·1 c.c. co-enzyme solution (p_H 7·4) were added to each and 0·25 c.c. methylene blue to tubes 1 to 5. After making up the volume in each case to 1·55 c.c. with distilled water, all the tubes were evacuated and immersed in the water bath at 37° C.

It can be seen that the extra inhibition by xanthine oxidase induced by the previous aerobic incubation (shown by the difference between the reduction times of tubes 2 and 3) does not occur to any appreciable extent if the previous incubation is carried our anaerobically (*cf.* tubes 2 and 5). If, however, methylene blue is present as an alternative hydrogen acceptor, oxidative destruction of the dehydrogenase can occur even during anaerobic incubation and the full inhibition by xanthine oxidase is obtained (*cf.* tubes 7 and 3). The fact that the inhibition in tube 5 is slightly greater than that in tube 2 may be due to exposure of the mixed enzymes to air during the setting up of the experiment, to traces of oxygen in the evacuated tube, or to traces of some hydrogen acceptor in the solutions themselves. This experiment has been repeated a number of times with different concentrations of xanthine oxidase, each time with similar results. It seems clear, therefore, that xanthine oxidase inhibits the activity of glucose dehydrogenase by bringing about an oxidative

destruction of the dehydrogenase. In parenthesis, it may be mentioned that this destructive action of xanthine oxidase probably accounts for the instability of solutions of the dehydrogenase made by the method first described (Harrison, 1931). Solutions of this dehydrogenase made by the later method (Harrison, 1933) have been found to contain much less xanthine oxidase and the solutions are much more stable.

To return now to the question of the structure of glucose dehydrogenase, the xanthine or Schardinger oxidase has been investigated as regards possible substrates probably more thoroughly than any other oxidizing enzyme, and the only substances which have found been to be oxidized by the enzyme are aldehydes and certain purines (Coombs, 1927). Having shown in the foregoing experiments that the oxidase brings about an oxidative destruction of glucose dehydrogenase, it seems reasonable to infer that the activity of the dehydrogenase is linked up with either an aldehyde or a purine group. In order to distinguish between the two possibilities, experiments were carried out to find whether the activity of the enzyme is depressed by the addition of aldehyde-binding substances. Sodium bisulphite was first tried and was found to produce a strongly inhibiting action on the oxidation of glucose by the dehydrogenase. The inhibiting action of M/100 $NaHSO_3$ at p_H 6·0 with different concentrations is shown in Table V. Each tube contained 0·4 c.c. enzyme-co-enzyme solution (p_H 6·0) equivalent to 0·6 gm. acetone liver, together with 0·25 c.c. M/2 p_H 6·0 phosphate buffer and 0·25 c.c. methylene blue. The total volume in each tube was made up to 1·55 c.c. with distilled water.

Table V.

No.	Glucose final concentration.	Time in minutes for decolorization of methylene blue.
1	0·032 M.	18
2	0·032 M. + M/100 $NaHSO_3$	62
3	0·065 M.	12½
4	0·065 M. + M/100 $NaHSO_3$	41
5	0·13 M.	11
6	0·13 M. + M/100 $NaHSO_3$	26
7	0·26 M.	12
8	0·26 M. + M/100 $NaHSO_3$	17½

It can be seen that the amount of inhibition produced by a given concentration of bisulphite depends upon the concentration of glucose. At low concentrations of glucose, the inhibition produced by bisulphite is relatively

large compared with that produced in high concentrations of glucose. Mann (1932, *b*) has shown that the optimum concentration of glucose for the dehydrogenase depends on the amount of co-enzyme present in the solution. It is clear from the above table that with a given amount of co-enzyme the optimum concentration of glucose is much higher in the presence of bisulphite than in its absence. The bisulphite and the glucose apparently compete for the same group on the enzyme. That the inhibiting effect of bisulphite is reversible is shown by the fact that if a solution of the dehydrogenase is precipitated at p_H 6·0 by ammonium sulphate in presence of M/100 $NaHSO_3$ and the precipitate is dissolved up in water, the enzyme is found to have lost little or none of its activity as compared with that of an equal amount of enzyme precipitated in the absence of bisulphite. The bisulphite compound with the enzyme is thus freely dissociated and the inhibition by bisulphite is readily reversible.

Table VI shows the inhibiting action of M/1000 $NaHSO_3$ on another preparation of glucose dehydrogenase and co-enzyme, and shows also that, unlike the inhibition by xanthine oxidase, the inhibition by bisulphite is not increased by previous incubation, the equilibrium evidently being attained rapidly.

Table VI.

No.	—	Time in minutes for decolorization of methylene blue.
1	Dehydrogenase + co-enzyme previously incubated	24
2	Dehydrogenase + co-enzyme + M/1000 $NaHSO_3$ previously incubated	42
3	Dehydrogenase + co-enzyme not incubated	22
4	Dehydrogenase + co-enzyme + M/1000 $NaHSO_3$ not incubated	44
5	Dehydrogenase + co-enzyme (control without glucose)	180

The enzyme + co-enzyme solutions (p_H 6·0) in tubes 1 and 2, with and without M/1000 $NaHSO_3$ respectively, were incubated aerobically at 37° C. for 20 minutes before adding glucose (0·13M.) and methylene blue, while the corresponding tubes 3 and 4 were not previously incubated. Each tube contained M/12 p_H 6·0 phosphate buffer.

The lability of the bisulphite compound made it appear less likely that much inhibition would be obtained with low concentrations of less powerful aldehyde-binding substances. No inhibition was obtained with M/50 semicarbazide or M/50 aniline at p_H 6·0 or 7·6. Aminoguanidine at p_H 7·0 produced a small

inhibition in concentrations of M/10 and M/50. Hydroxylamine (M/50) at p_H 6·0 and 7·6 allowed the decoloration of methylene blue to take place up to a certain point after which the solution always remained blue even on very prolonged incubation. It appears likely that after the methylene blue has become partially reduced and a certain reduction potential has been reached in the solution, the hydroxylamine itself acts as a hydrogen acceptor and becomes reduced instead of the methylene blue. Phenyl hydrazine could not be used in these experiments as it reduces methylene blue rapidly by itself.

If sodium bisulphite inhibits by virtue of its power of combining with aldehyde groups, it would be expected that no inhibition would be produced in slightly alkaline solutions, since it is well known that the aldehyde-bisulphite compounds are readily decomposed in weakly alkaline solutions. On carrying out parallel experiments with the same solutions at p_H 6·0 and p_H 7·6 (allowing for the controls with bisulphite but without glucose) it was found that while a large inhibition on the action of glucose dehydrogenase was produced by M/100 $NaHSO_3$ at p_H 6·0, no inhibition was produced at p_H 7·6.

Owing to the freely reversible nature of the bisulphite inhibition, it is not possible to obtain such definite evidence as to the mode of action of bisulphite as was obtainable with the irreversible inhibition produced by xanthine oxidase on glucose dehydrogenase. It is not claimed that the bisulphite experiments in themselves constitute proof of the aldehyde nature of the glucose enzyme. Taken in conjunction with the experiments with xanthine oxidase, however, showing glucose dehydrogenase to be either an aldehyde or a purine, the bisulphite experiments render it extremely probable that the activity of the glucose dehydrogenase is associated with the presence of an aldehyde group.

As pointed out by Willstätter (1932), the constitution of an enzyme cannot be determined in the same way as the structure of simpler substances such as vitamins and hormones. It seems likely that the principle of using enzymes known to act on specific substrates, an example of which is given in this paper, may be of more general use in elucidating the structure of the active groupings of other enzymes.

Part of the expense was defrayed by a grant from the Government Grant Committee of the Royal Society.

Summary.

Xanthine oxidase (or the Schardinger oxidase) can bring about complete inhibition in the oxidation of glucose by glucose dehydrogenase. It is shown

that this inhibition is caused by an irreversible oxidative destruction of the glucose dehydrogenase by the oxidase.

Since the xanthine or Schardinger oxidase acts only on aldehydes and certain purines, it is inferred that glucose dehydrogenase contains either an aldehyde or purine group.

From the fact that the aldehyde reagent, sodium bisulphite, reversibly inhibits the activity of the dehydrogenase in slightly acid solution, even in concentrations of M/1000, it is deduced that the activity of the enzyme glucose dehydrogenase is associated with the presence of an aldehyde group.

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Appetite and Choice of Diet. The Ability of the Vitamin B Deficient Rat to Discriminate between Diets Containing and Lacking the Vitamin.

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It has often been assumed that animals given a suitable free choice of diets are able to select satisfactorily according to their individual nutritional needs. However, no systematic work has hitherto been undertaken to ascertain how far this is true, nor have we any knowledge of the mechanism involved in the choice. Lusk (1928), in his well-known text book on nutrition, refers to the observation of a German worker (Tscherkes, 1923) that fowls suffering from polyneuritis will search out green food and refuse to accept grain. This he describes as due to the "triumph of instinct." The similar eagerness of vitamin B deficient rats to consume diets containing the vitamin must, we imagine, have been noticed by observant workers. It is, however, unsatisfactory to allow the matter to be ascribed vaguely to some unexplained—if not inexplicable—"instinct." If animals are in fact able to distinguish between foods containing and deficient in the vitamin, what is the nature of the process involved? Has the animal some means, as by taste or smell, of recognizing the vitamin *per se*? If so, it would imply an ability to detect a constituent amounting to no more than perhaps 1 part in a million of the food. If not, by what means is the rat able to recognize the vitamin-containing food? And what are the limitations to such powers of recognition? These are some of the questions which one sought to answer. An effort to analyse these phenomena was begun in this laboratory in 1928, and in the present paper are summarized the principal results reached during the period 1928-1931.* To anticipate a main conclusion it may be said at once that we have obtained good evidence that the behaviour of the animal is due not so much to instinct as to

* Miss Hargreaves took part in the investigation during the winter of 1928, Miss Clay during the session 1929–30, and Mr. Ward in the preliminary and again the final stages.

experience, i.e., of the beneficial effect produced by a particular foodstuff. We believe that this factor of experience plays an important part in determining dietary preferences in general, and it will certainly have to be taken into account in future work on that hitherto neglected subject, the psychology of appetite.

Apart from the reference quoted above we know of no previous literature relating to the original point of departure of our enquiries. Recorded work on the whole question of the free choice of diet is almost equally scanty. Osborne and Mendel (1918), working on the comparative food values of different proteins, thought that rats " as a rule ate more of the adequate than of the inferior food " ; but Beadles, Braman and Mitchell (1930), on the contrary, could find " no support for the assumption that the more complete of the two rations is consumed in greater amount." Nevens (1928) noted that cattle given a free choice of limestone, bone meal and salt, offered as supplements to an inadequate diet, took only insignificant amounts of the first two ; while Kon (1931) found that rats might fail to take enough protein even to keep them alive, when allowed to choose their own allowance of carbohydrate, protein and salt mixture.

General Methods.

Young growing rats have been used exclusively in the work described below. Several hundred animals have been used in all, and since in many cases each rat was offered simultaneously the choice of a considerable number of different diets, the intakes of which were to be determined daily, a special type of technique had to be worked out. After a little experience the following method was adopted, and has proved to be very satisfactory. A moisture-free basal diet (vitamin-free casein 20 per cent., starch or sugar 55 per cent., arachis oil 20 per cent., salt mixture 5 per cent., cod liver oil 2 drops per rat daily) is used, to which various supplements to be tested are added. The basal diet contains a high percentage of the oil, so as to give it a tacky consistency and prevent errors caused by animals scattering their food. Each different diet is put in a separate tall, narrow, glazed porcelain jar, cylindrical in shape and measuring 6 cm. high by 3—3·5 cm. in diameter (pharmacists' " ointment pot "). At the beginning of the working day the diet is placed at the bottom of the jar and pressed well into position by means of a glass crusher (rod with perpendicular glass disc at bottom). The total weight of the jar plus contents is determined to the nearest 0·2 g. on an Avery automatic weighing machine, with scale marked in 25 g. by 0·2 g. The various pots, each containing a different diet,

are then exhibited in a row, along the inner side of the cage. (Each rat, of course, is kept in a separate cage, and all experiments are performed in duplicate on a batch of four or more rats.) Pots are kept in place by semicircular steel springs securing them to the upright metal bars of the cage. In most experiments the relative positions of the pots was varied each day. The following morning the pots are removed, and reweighed on the automatic machine. The loss in weight for each pot gives directly the weight of dry food eaten; it is unnecessary to know the actual weight of the pot or of the diet placed in it at the beginning of the day or remaining at the end. Owing to the narrow shape of the pot the rat has to thrust his head deep down into it to obtain his food, and owing to the sticky nature of the diet there is little danger of it being removed from the pot and left uneaten. Any animals showing a propensity to scatter their food are disqualified from taking part in the experiment.

Results. Part I.

Experiments 1 *to* 4.—*Comparison of Choice made by Deficient as Contrasted with Non-deficient Rats.*

Fig. 1 represents (above) the weight curves, and (below) the different foods offered and the amounts eaten by each of a group of four duplicate rats. It will be noted that at the beginning of the experiment the rats are restricted to a diet deficient in vitamin B. After about a week appetite begins to drop markedly and the animal loses weight rapidly. After about 3 weeks, the rat, now suffering from fairly severe vitamin B deficiency, is offered the choice of three diets, viz., (1) the original basal diet, (2) the same diet flavoured with bovril (5 per cent.), and (3) flavoured with marmite (5 per cent.) The last mentioned alone contains vitamin B, and in this experiment care is taken that there is no more than a bare sufficiency of it. The depleted rat is found to select the marmite diet practically exclusively, insignificant amounts of the other two being taken. In contrast, control rats which have not been so depleted, when offered the identical choice of "basal," "bovril" and "marmite" diets, are found to eat all three indiscriminately (fig. 2). Towards the end of the experimental period, however, when they too are beginning to feel the shortage of the vitamin (not sufficient being obtained by eating an assortment of all three diets) they tend likewise to go over to the "marmite" diet.

This experiment has been confirmed repeatedly using various other sources of vitamin B in place of marmite, but always with the same result—an almost quantitative selection of the adequate food by the depleted rats. The

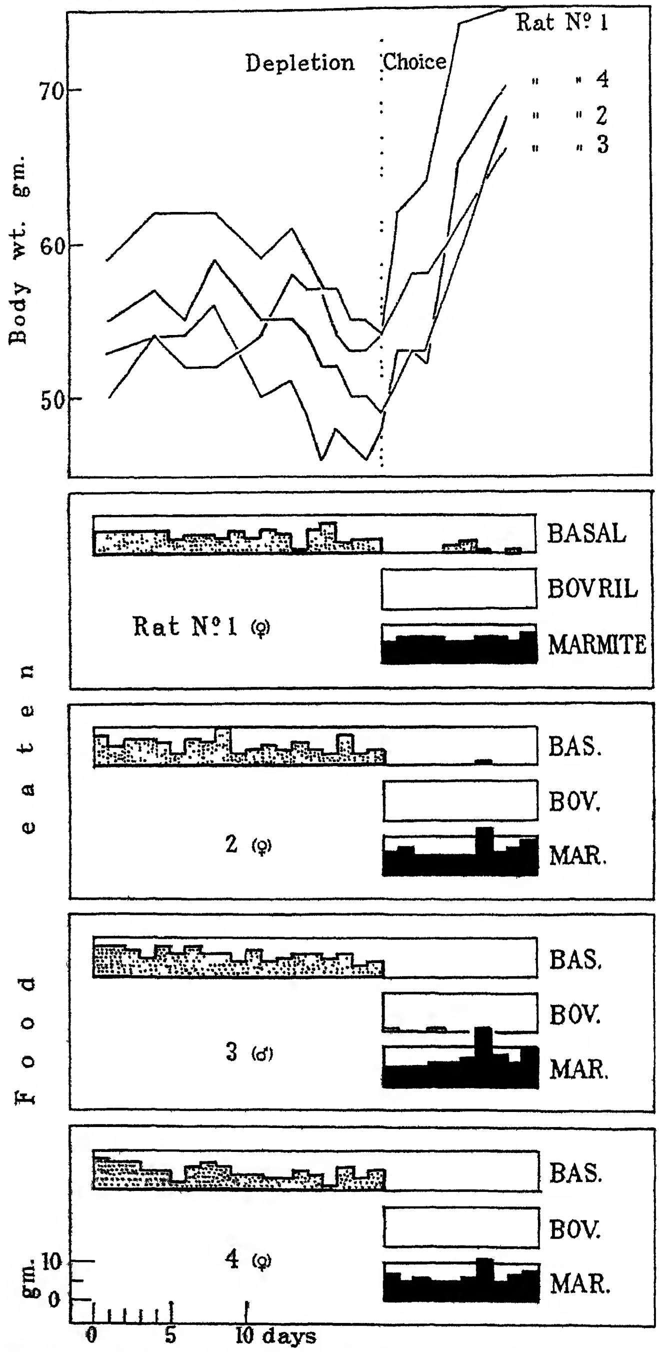
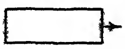


Fig. 1.—Preference of vitamin B depleted rat for diet containing the vitamin (*cf.* fig. 2)
[In this and subsequent diagrams :—


→ indicates that the diet was *offered* during the period shown.


→ represents the amounts of the diet *eaten* day by day.


= diets containing adequacy of the vitamin.


= diets devoid of the vitamin.]

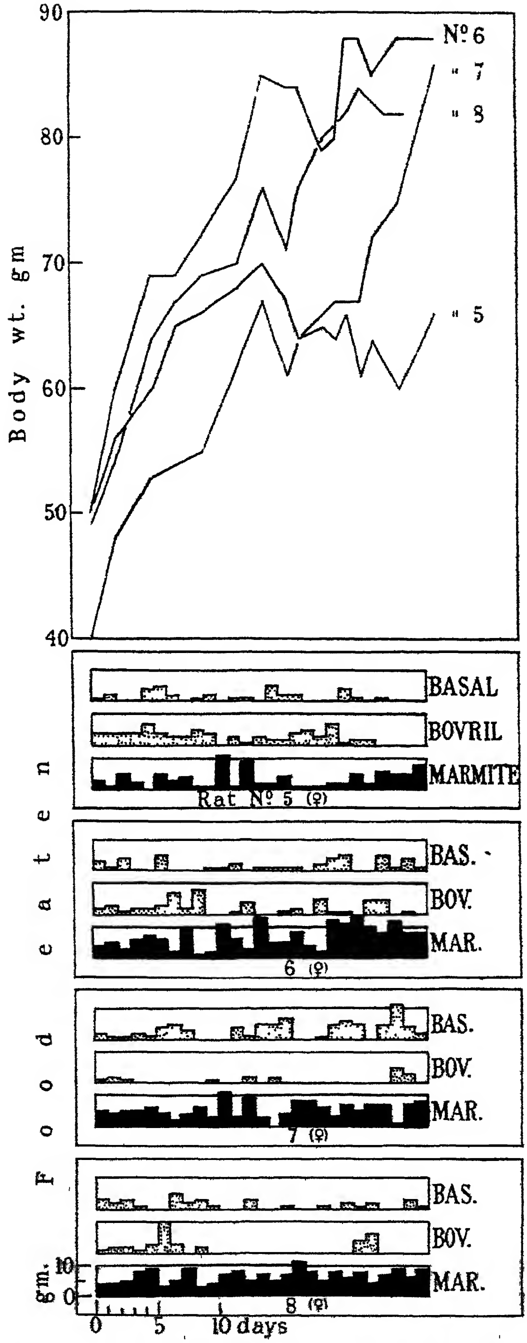


FIG. 2.—Lack of preference in rats not depleted (cf. fig. 1).

objection may perhaps be raised that these results are affected by such factors as palatability, the monotony of a given diet, or the novelty of a new one, but these factors have been fully controlled, as described in later experiments.

A point we wish to stress as particularly significant is the *immediate rapid recovery* of the deficient rats, fig. 1, as soon as they start eating the vitamin-containing diet. This is shown graphically by the growth curve and the immediate restoration of appetite, but there is likewise a marked improvement in the animal's general condition, especially (as there is good evidence to show) in the rapid return to normal of alimentary conditions, the avitaminosis being characterized by defective secretion of digestive juices, and by gastric and intestinal distension or atony.

The controls, fig. 3, incidentally give a further demonstration of how immediate is the recovery of the deficient animal once it is taken off the vitamin-free basal diet and given the vitamin-containing diet. The "bovril" diet failed to aid recovery, or to protect against the symptoms of the avitaminosis, figs. 3 and 4.

Experiment 5.—*Choice between Various Levels of Partial Vitamin B Adequacy.*

This experiment, fig. 5, lies rather aside the main sequence of argument of the paper, but may be mentioned briefly here. Rats "run out" of vitamin B and then offered the choice of two diets, one containing an insufficiency of marmite (1 per cent.) the other a bare adequacy (5 per cent.), chose the latter almost exclusively. When next offered the choice of four diets containing 1 per cent., 4 per cent., 5 per cent., and 6 per cent. of marmite, they tended on the whole to take the diets richer rather than those poorer in marmite (vitamin B). One animal, it is true, chose the 5 per cent. rather than the 6 per cent. level, but there was good evidence of an ability to discriminate on the whole in favour of the higher rather than the poorer levels; only when the various levels were too nearly matched was the rat unable to distinguish between them.

Experiment 6.—*Absence of Discrimination when the Vitamin is Provided Separately.*

The purpose of this experiment was to discover whether rats would cease to show the preference for the vitamin-containing diet when a separate source of vitamin B was given independently. The separate source of vitamin B used was orange juice, given *ad lib.* in place of the usual drinking water. (This

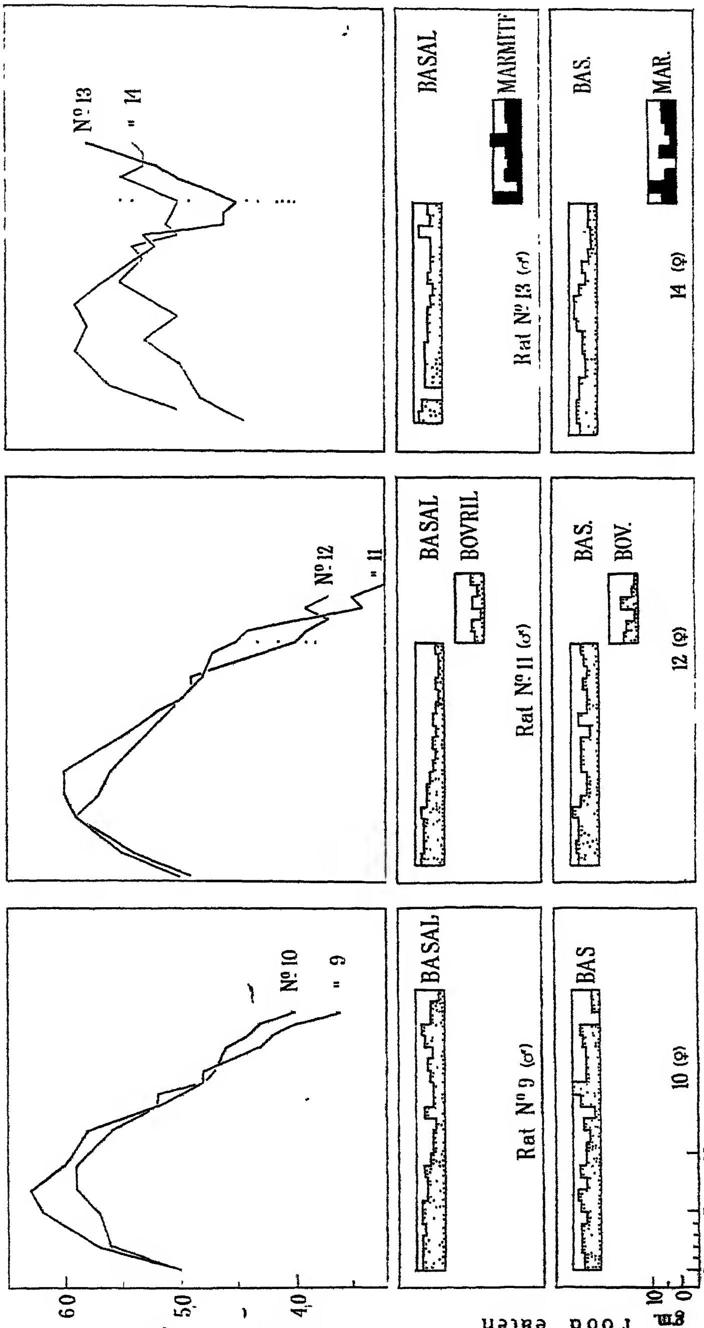


Fig. 3.—Controls to fig. 1, showing effectiveness of "marmite" diet in curing the avitaminosis and ineffectiveness of "basal" and "bovril" diets.

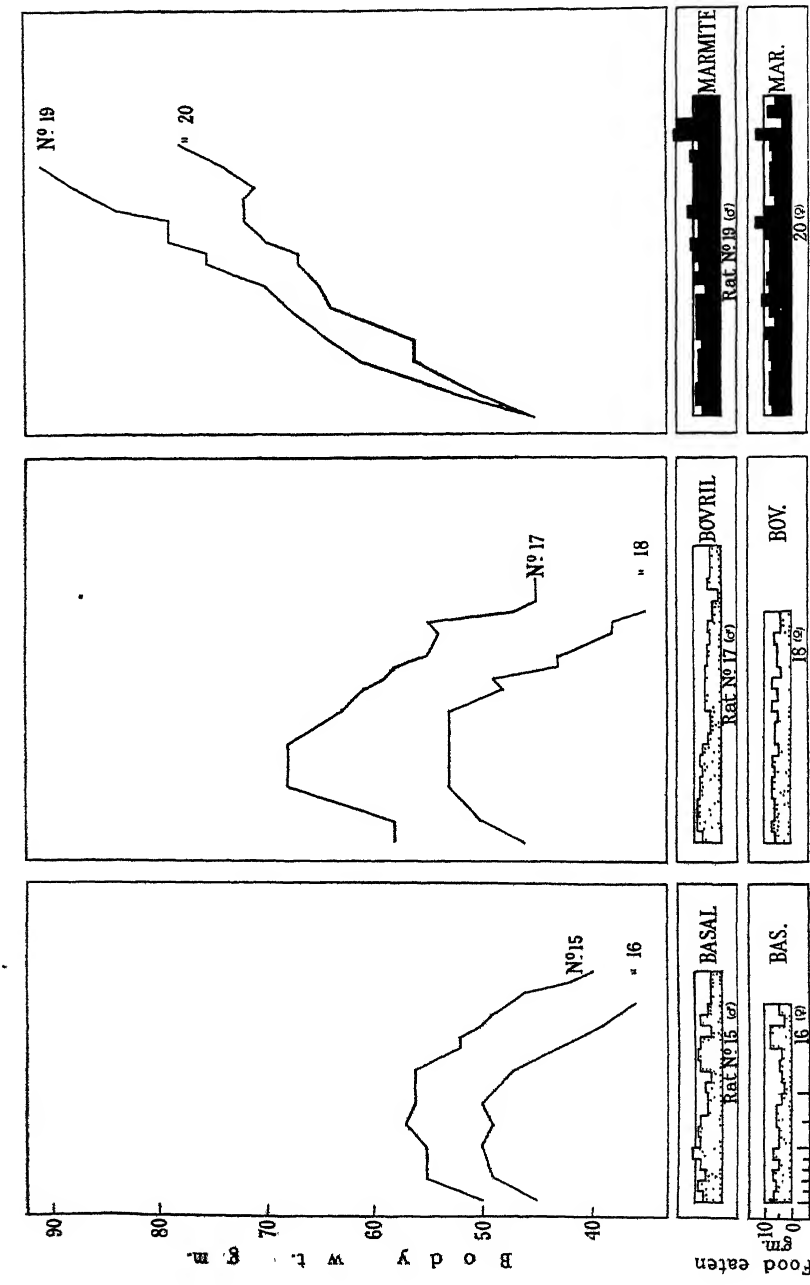


Fig. 4.—Controls to fig. 2, to show adequacy of "marmite" but not of "bovril" or "basal" diets to support continued growth.

is supplied in a long necked, narrow lipped inverted flask.) It will be seen, fig. 6, right hand, that, in contrast with the results obtained in experiment 1,

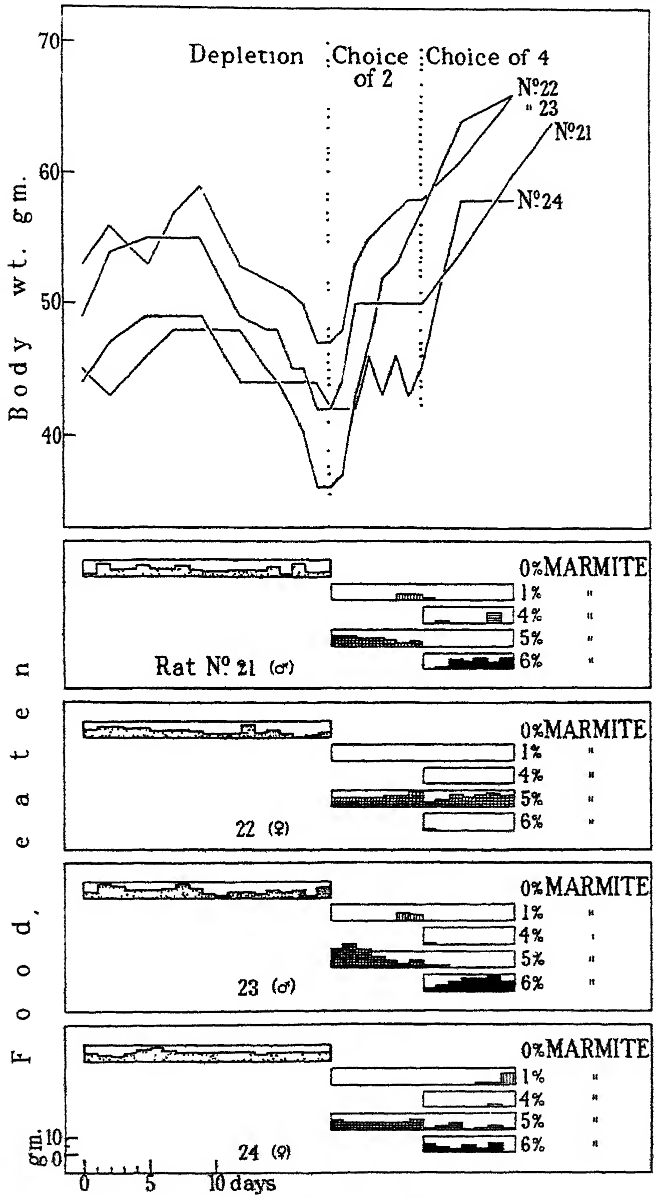


FIG. 5.—Depleted rats offered diets containing various levels of vitamin B.

animals now showed no preference for the "marmite" over the "bovril" diet, after having been on the basal diet for the usual period. In this experi-

ment the orange juice was given from the start. But on the other hand, when the orange juice was not given until the animals were already depleted fig. 6,

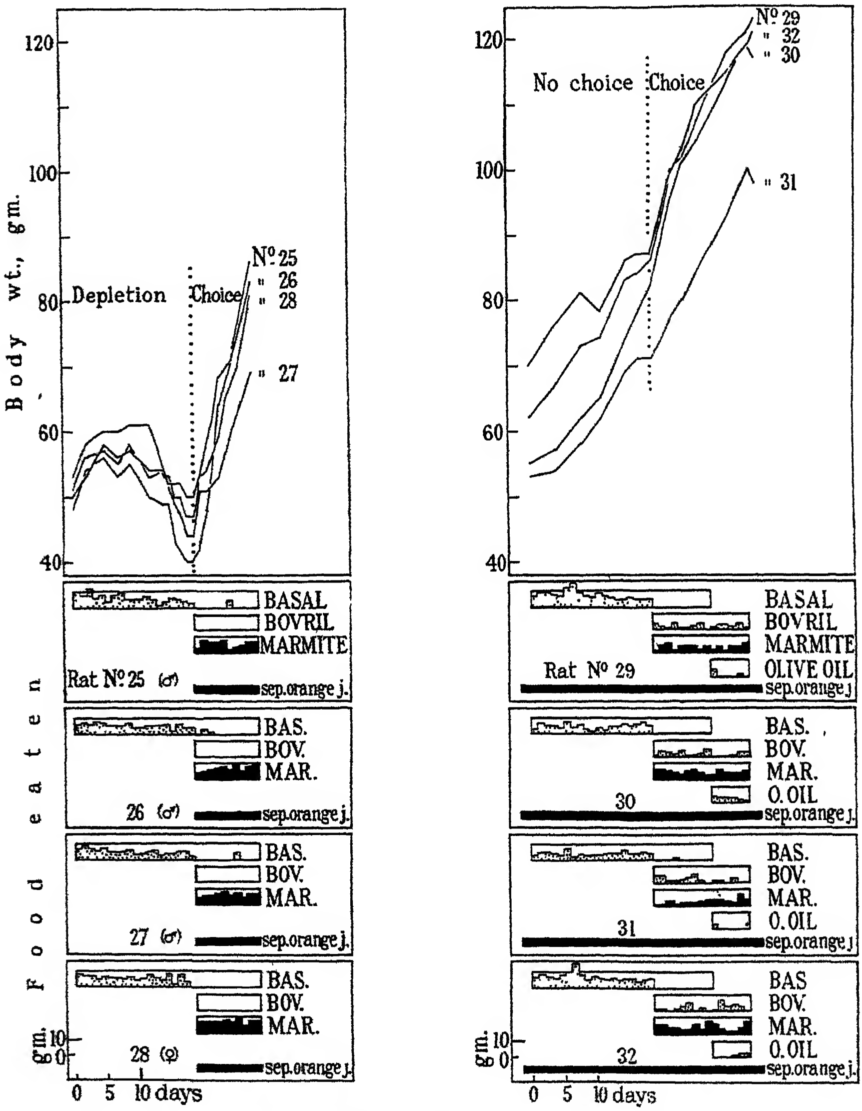


FIG. 6.—On right: separate vitamin B given from start—no preference for the vitamin B containing diet. On left: no separate vitamin B during depletion—preference for the vitamin-containing diet.

(left hand), there was the usual preference for the "marmite" diet. Here the appearance was as though the depleted animals were determined to obtain their

full allowance of the vitamin (of which they stood in such severe need), by taking both the marmite and the orange.

The experiment under discussion furnishes a good illustration of a point to which passing reference may be made; that is, the tendency which an animal may exhibit—but under certain conditions only, as will be apparent later—to prefer the novelty of a new diet to the monotony of an old one. The four animals represented on the right hand chart, fig. 6, after about 3 weeks on the basal diet preferred to try the new "marmite" or "bovril" diets as soon as given the opportunity. This factor has been further studied in a later series of tests and it will be shown that under other circumstances an animal actually prefers to remain on the one same monotonous diet, when it is in need of the vitamin and the new diets are devoid of it.

Experiment 7.—Use of other Sources of Vitamin B, and Vitamin B_1.

Fig. 7, left, gives one example from among many experiments that have been carried out, in which an alternative source of vitamin B (2·5 per cent. of dried yeast) was used in place of marmite. It will be seen that after depletion the animal makes the usual choice of the diet containing the bare adequacy of the vitamin in preference to a vitamin-free diet. Similar results were obtained with various other sources of vitamin B, *e.g.*, orange juice; other inactive flavouring materials, such as olive oil, also being used in place of the bovril, etc., for the vitamin-free diets. But here once again when the vitamin was *given separately* from the start in sufficient amount no preference was shown for the vitamin-containing diet (the experimental charts, closely similar to figs. 1 and 6, etc., are not shown).

In an analogous set of tests the rat was depleted only of *vitamin B_1*, vitamin B_2 having been allowed as 1·5 c.c. of autoclaved marmite per rat per day, and it was found that a similar choice was made by the depleted rat for a diet containing vitamin B_1, such as wheat germ, in preference to one devoid of it.

We have included, fig. 7, right, the charts of two animals who behaved rather exceptionally in hesitating longer than usual before finally going over exclusively to the vitamin-containing diet—2·5 per cent. wheat germ. Possibly this had to do with the fact that they had been run out of the vitamin less far than usual, and so were in less severe need for it and gained less stimulus from its consumption.

In concluding this section, it may be pointed out that in all the experiments so far recorded no more than a bare adequacy of the vitamin was incorporated in the one vitamin-containing diet, which the vitamin depleted animal was

found to select exclusively. If instead too liberal an allowance were included (a fuller discussion of this point will be given later) it was found that the rat,

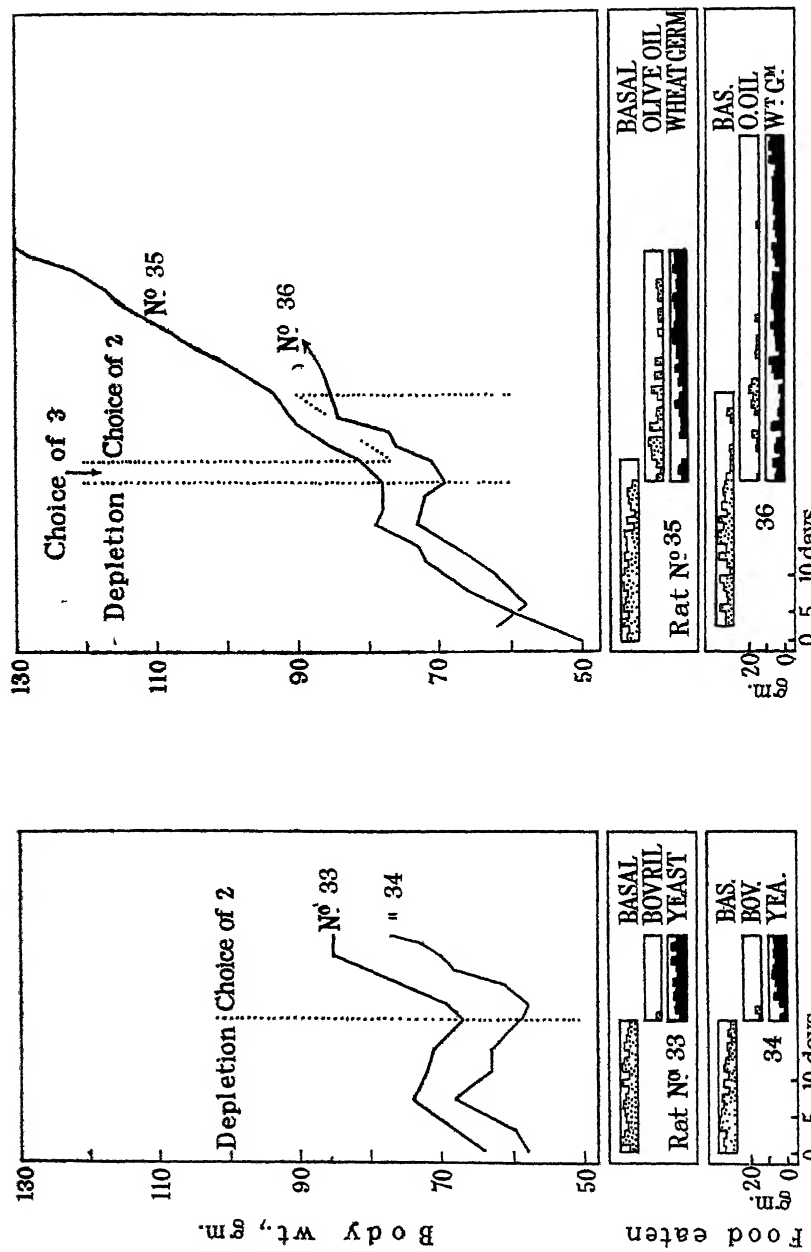


FIG. 7.—Preference of depleted rats for diets containing vitamin B (or B_1) in the alternative forms of yeast (on left,) or of wheat germ (vitamin B_2 provided) (on right).

now being able to satisfy his full needs and more, might after a time cease to restrict himself so exclusively to this one diet.

PART II.

Experiment 8.—*Failure to Select when the Choice is Sufficiently Large. Ability to be "Educated."*

In the experiments thus far described the depleted rat was offered only one or two vitamin-free diets in addition to the one diet containing the vitamin, and under these circumstances was able to select the vitamin-containing diet. We now turn to a description of experiments in which a much larger choice of the various differently flavoured vitamin-free foods was offered, together with the one containing the vitamin. A typical experiment is shown in fig. 8, left. The rat is depleted as before, but the choice of diets offered after depletion is made so large that the animal has difficulty in selecting the vitamin-containing diet. If at this stage the vitamin-containing diet alone is offered for 2 or 3 days—" education " period—the animal makes a rapid recovery and regains his appetite, and when he is once more confronted with the same large choice of diets as before, he is now able to make the choice correctly—presumably because of the experience gained during the education period. On the other hand, some animals may succeed in discovering the vitamin-containing diet unaided after a short period even from among a very large choice, fig. 8, right. Such animals once having discovered the diet containing the vitamin, on which they are able to recover, are found to continue to take it exclusively, preferring apparently to remain on the monotonous but beneficial diet rather than indulge in sheer variety at the expense of the discomforts of avitaminosis.

Experiments 9 *and* 10.—*Further Studies on "Education" with Larger Choice of Diets.*

In fig. 9, left, we have given another instance of a depleted rat being at first unable to select the vitamin-containing diet, the choice of vitamin-free diets offered simultaneously being very large. After " education " the choice was made with no difficulty, although no less than 10 different diets were offered. This experiment is only typical of many recorded. On the right-hand side of the figure, we give an instance of a rat who was beginning to discover for himself the vitamin-containing food and to thrive on it before the institution of the " education " period, notwithstanding the difficulty of the large choice offered. In fig. 10 a different source of vitamin B, orange juice, is used in place of marmite, and the procedure adopted differs somewhat in detail. It will be seen that after depletion each rat was able to select the vitamin B containing diet

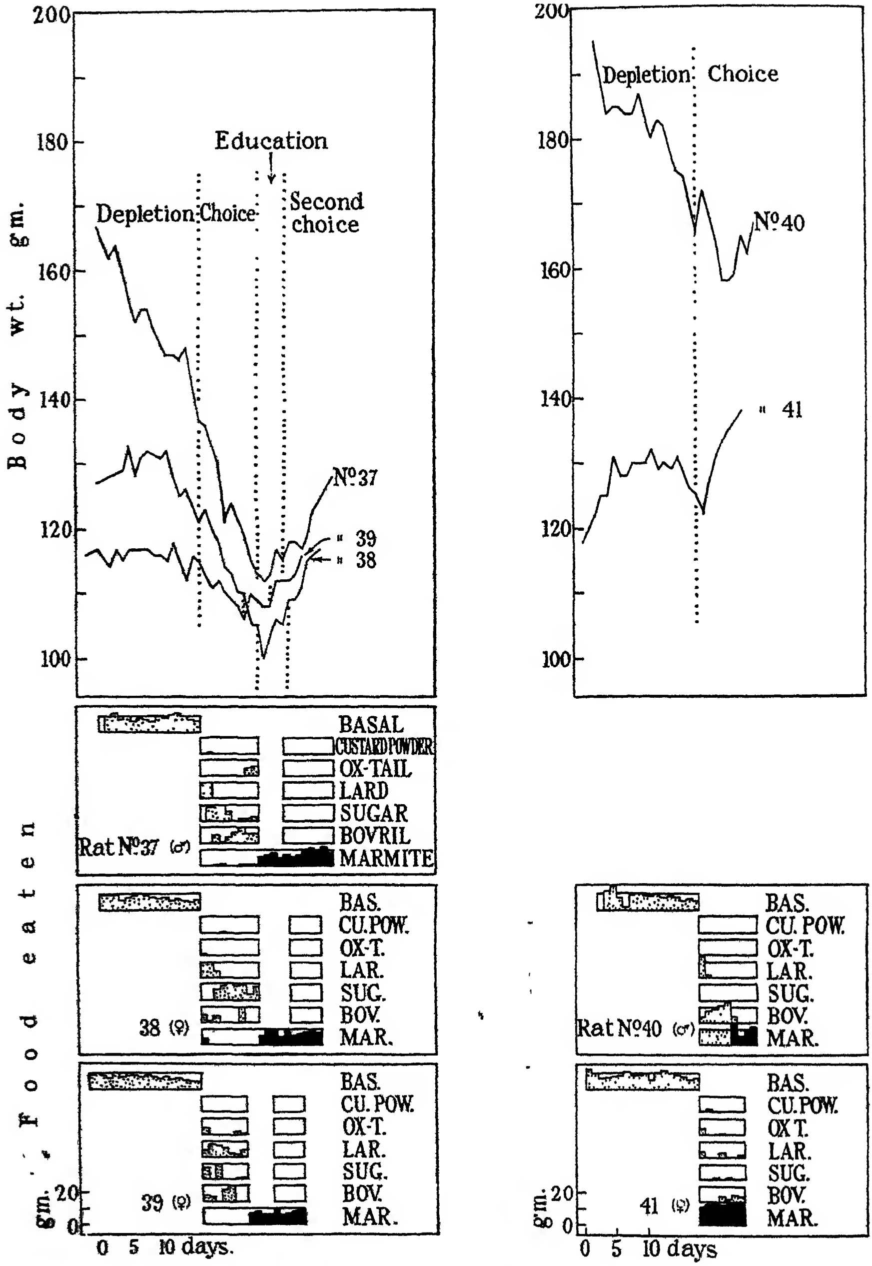


FIG. 8.—On left: large choice—majority of rats fail to select correctly until after "education." On right: examples of rats succeeding in making the correct selection unaided.

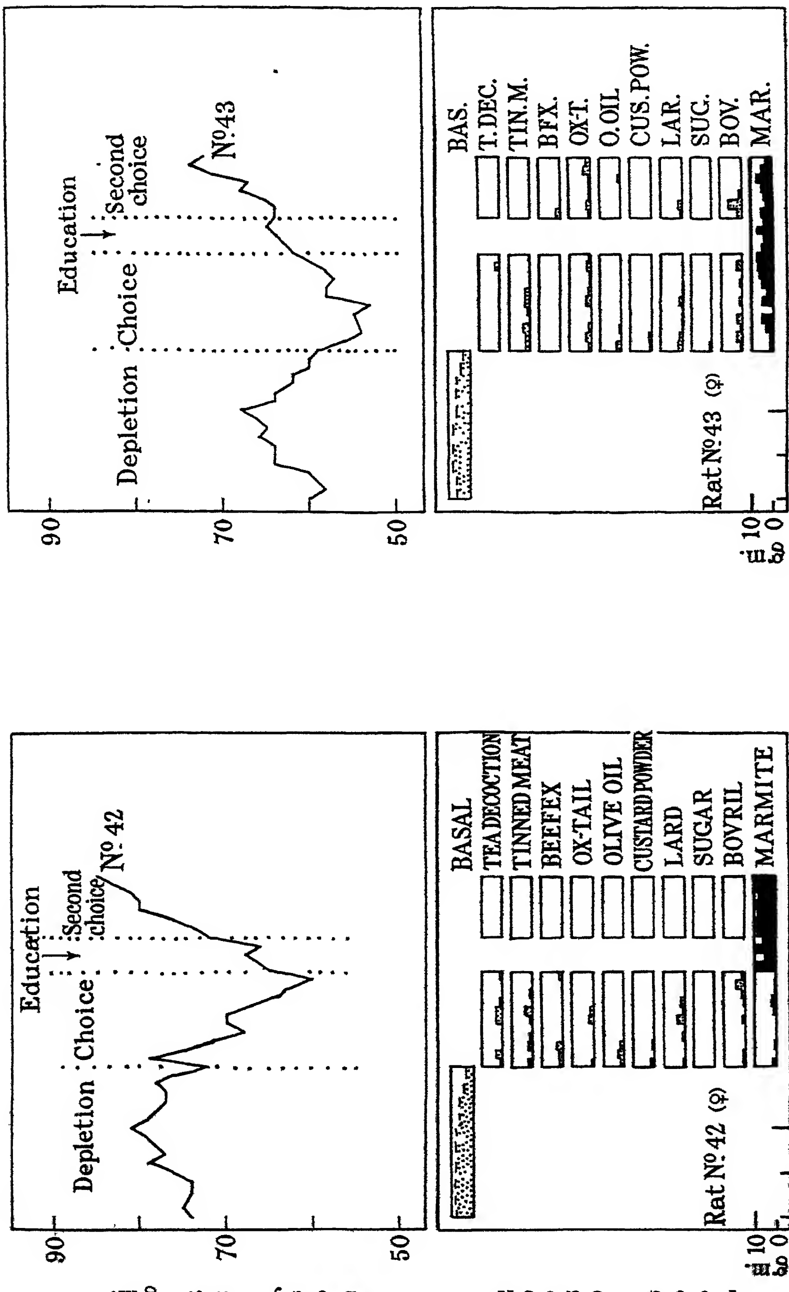


Fig. 9.—Still larger choice—selection of the vitamin-containing diet by the depleted rat after "education," or after experience of its beneficial action.

from a choice of three diets, and when the choice offered was later further enlarged he still continued to prefer the orange concentrate to the newly offered diets.

Experiments 11 *and* 12.—*Experiments to Control the Factors of "Monotony" and "Novelty" in the Diet.*

The experiment just described, fig. 10, shows that a depleted rat may prefer to restrict himself to one monotonous diet to which he has been "educated" and on which he is thriving, although other alternative diets are offered which would be accepted indiscriminately by non-depleted rats. A further examination and controlled experiments on this point were necessary. Figs. 11 and 12 may be considered together in this connection. From fig. 11 it is seen that a rat depleted of vitamin B and "educated" to marmite will continue to eat the beneficial diet virtually exclusively, notwithstanding the increasing choice becoming available as fresh diets are in turn offered. The control experiment, represented in fig. 12, makes it clear that it is not simply a matter of the rat continuing to eat any diet to which he has been made accustomed. This test, fig. 12, is run on parallel lines to experiment 11, the difference being that here it is attempted to "accustom" the depleted rats to various *vitamin-free* instead of to vitamin-containing diets. It will be seen at once that the rats fail to continue eating the given *vitamin-free* diet when a larger choice becomes available, and the contrast with their behaviour when educated to a *vitamin-containing* diet is readily apparent.

A further control of the type of experiment described in this section is shown in fig. 13. On the left is represented a group of rats who, following the usual procedure, were depleted, then offered a choice, then educated, and then re-offered the same choice. The identical procedure was adopted with a control group of rats (on right) who, however, were *fed with vitamin B separately* as orange juice. Under the latter conditions the education period was without effect, the animals being able to thrive on any of the diets gained no special experience or stimulus from the "education" period, and so continued to eat all diets indiscriminately.

In other words, provided it has had the experience of the beneficial effect of a given diet containing the vitamin, the vitamin-depleted rat will prefer to stay on this diet notwithstanding the monotony of it; while, on the other hand, it will not stay exclusively on any *vitamin-free* diet, but will "test" other diets in turn. Rats not depleted, and so not needing the vitamin and

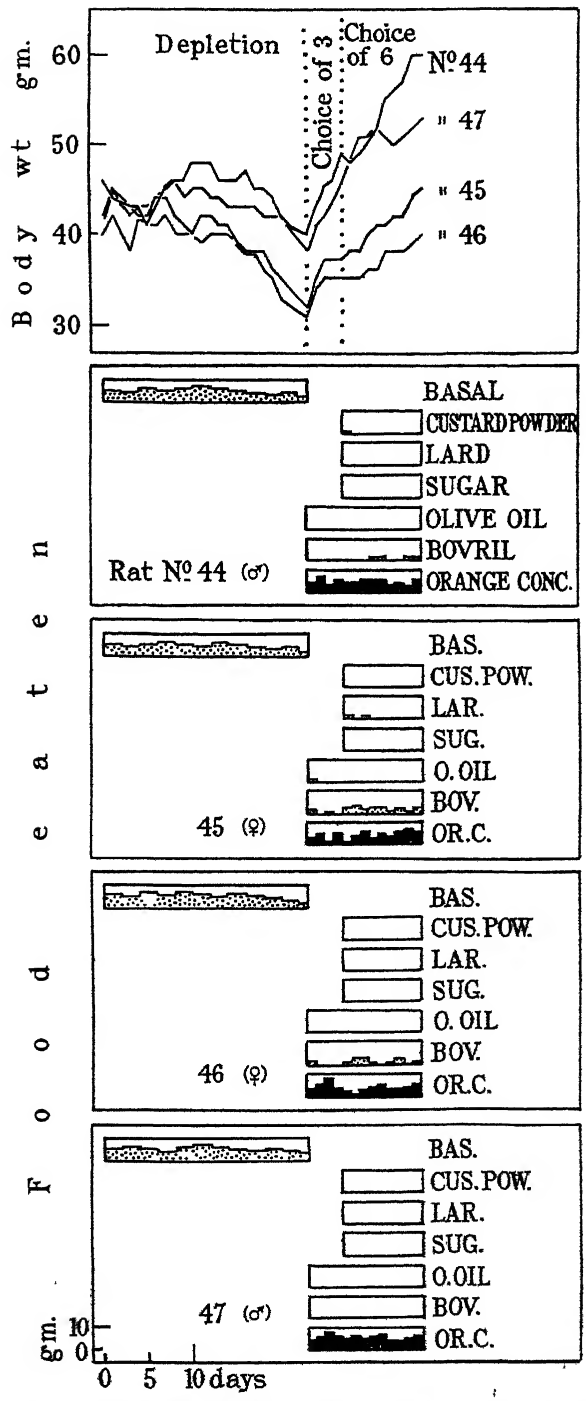


FIG. 10.—Use of an alternative source of the vitamin (orange juice), and continued preference for it notwithstanding the increasingly large choice of alternatives offered.

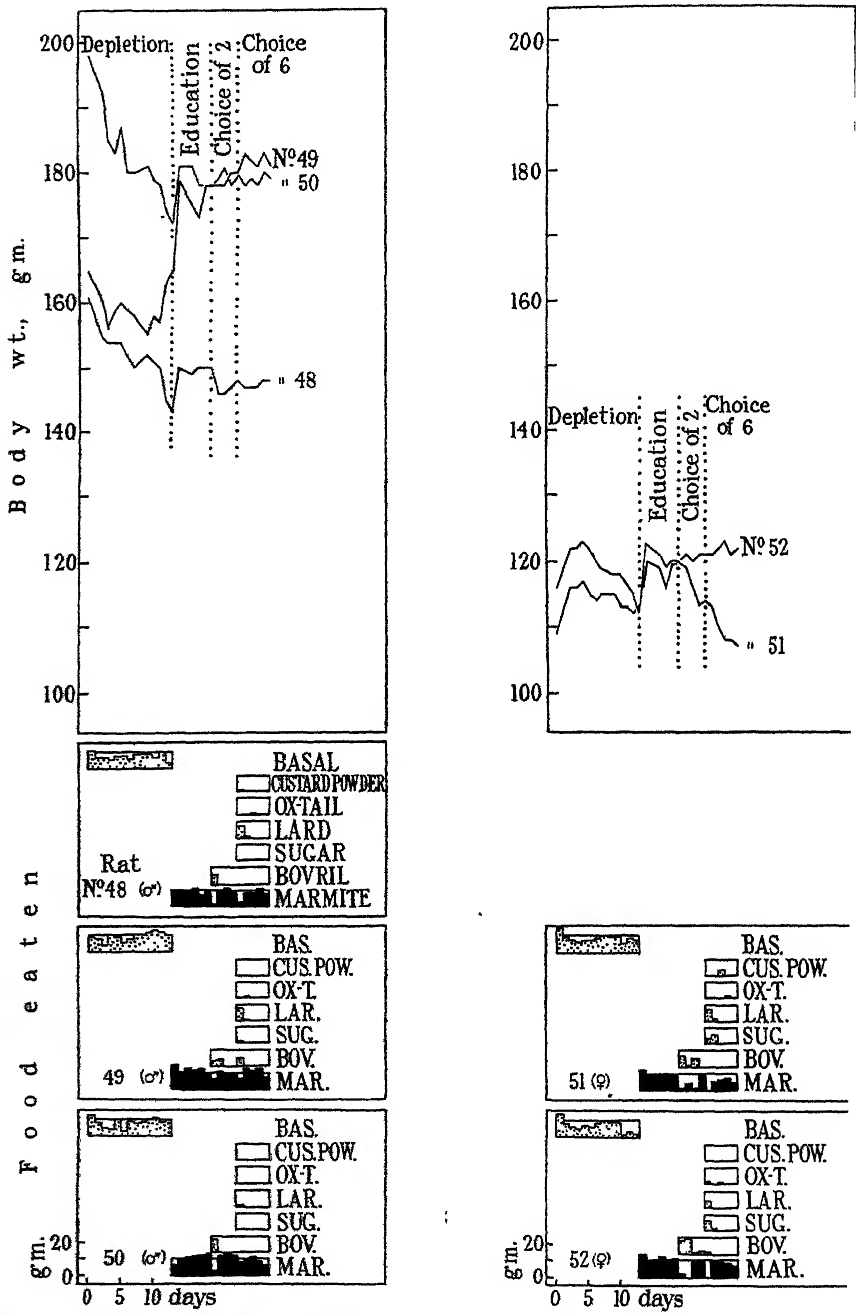


FIG. 11.—Selection by depleted rat of a "monotonous" vitamin-containing diet, in prefe ence to various new, but vitamin-free, diets offered.

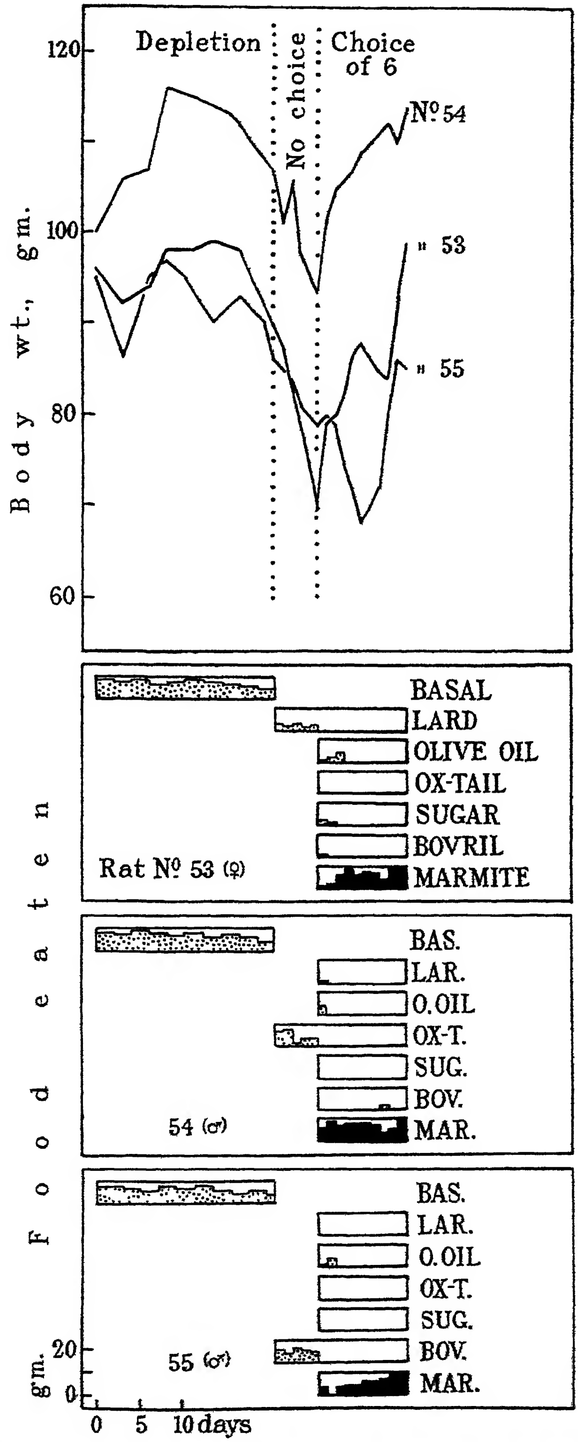


FIG. 12.—" Monotony " control to fig. 11 : failure of depleted rats to select diet to which they are accustomed but which are devoid of the vitamin.

not immediately stimulated by the vitamin-containing diet, will eat all diets indiscriminately.

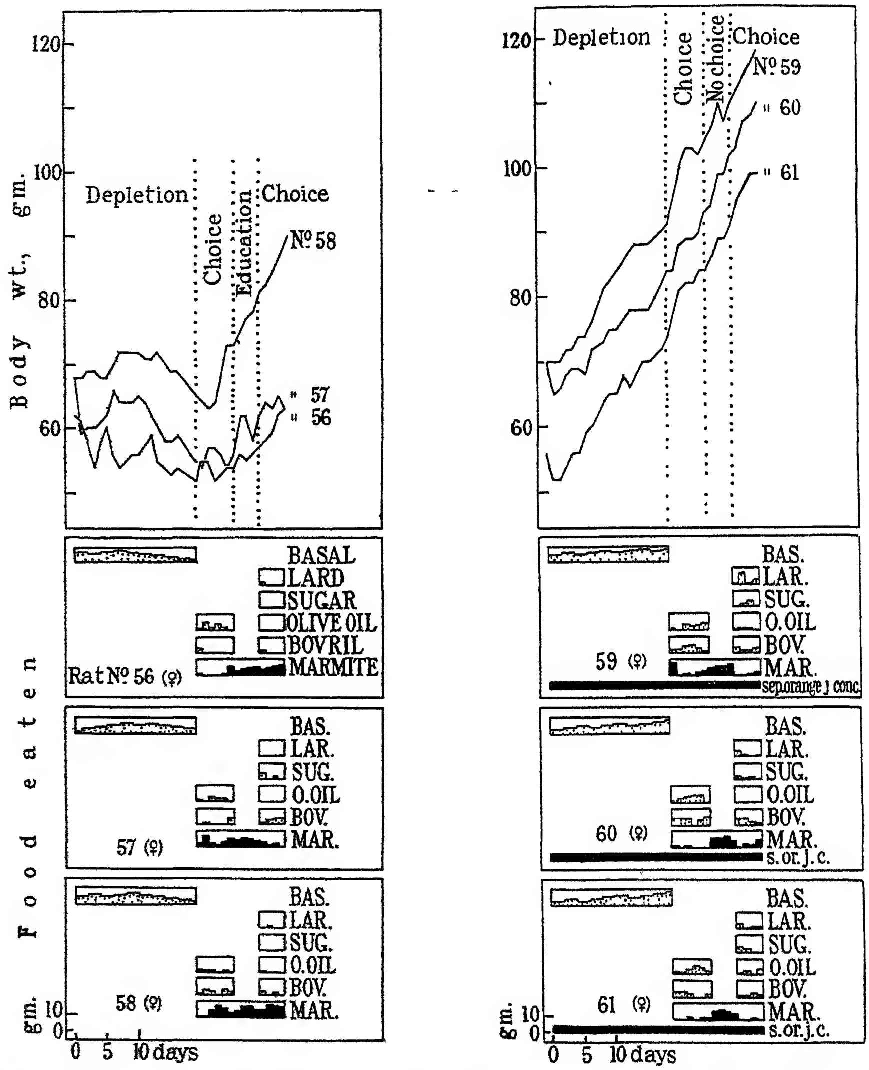


Fig. 13.—Controls to show ineffectiveness of "educational" period when vitamin B is given separately as orange juice (on right).

Part III.

The experiments already described strongly suggest that it is the experience of the immediate beneficial action of the vitamin-containing diet which causes the depleted rat henceforth to discriminate in its favour. We must assume

that the rat associates the pleasant sensations incident to his recovery with some characteristic or other (smell, taste, appearance) of the diet in question. So he comes to exhibit a special preference for a diet which under normal circumstances would possess no special attraction for him. Further experiments were devised to test the hypothesis.* In the first place it was shown that depleted rats had difficulty in discriminating between a vitamin-containing and a vitamin-deficient diet when the two were made very much alike, *e.g.*, when the vitamin was added no longer as a natural food source (as in the experiments described up to this point) but as a minute amount of a highly active concentrate, the vitamin-deficient diet being made identical except for the presence of the same amount of concentrate previously rendered inactive. That is, the rat is not able to distinguish vitamin B *per se* in diets, but rather it would appear that the recognition is for some separate distinctive characteristic in that particular diet which has produced the beneficial effect. This conclusion is strikingly confirmed in the following experiment which is perhaps the most important discussed in the present paper.

Experiment 14.—*Depleted Rats Misled to Eat a Deficient Diet.*

A group of rats was first depleted of vitamin B_1 in the usual way (Stage 1: "depletion period"—see fig. 14, rat No. 62). They were next "educated" to a diet which contained a bare adequacy of vitamin B_1 in the form of a highly active concentrate,† and to which a distinctive character had been lent by flavouring it with 5 per cent. bovril (Stage 2: "education" period). Following this, the usual chôice period followed; and the rats were shown to choose this "bovril" diet—to which they had been educated—to the exclusion of the three new diets offered, devoid of vitamin B_1 and flavoured with cocoa, or lard, or with no additional flavouring (Stage 3: choice period). The

* Good evidence that the sense of smell was generally the special sense factor principally involved was obtained in the experiments already recorded. Thus observations were made on rats which after depletion were selecting the vitamin-containing diet and rejecting the other alternatives offered. It was noted that as each morning the same choice of diets was placed before the rat he would first give a cursory sniff at each in turn and then proceed to eat the vitamin-containing diet—as though recognizing it as the one which had been responsible for his cure. Changes in the relative position of the several diets seemed to have but little or no effect on his choice, or on the ease of recognition. How far the other special senses can become involved is, however, a matter for further study. But it is evident that the technique described in this work provides a new method capable of wide application for studies on conditioned reflexes and learning.

† The basal diet contained sufficient vitamin B_2, as autoclaved yeast.

special feature of the experiment begins at this point. The active vitamin concentrate is transferred from the "bovril" to the "cocoa" diet (Stage 4: "deception" period). It is found that the rat continues to eat the "bovril" diet, which is no longer active but to which he had been "educated" and which had been associated with the improvement in his condition. Although the rat fails to thrive on this bovril diet, the urge to eat it is so strong that he may continue to choose it exclusively for a considerable period, losing weight and suffering all the ill-effects resulting from vitamin B_1 deficiency. At any time now, however, the rat may readily be "re-educated" to select whichever diet now contains the vitamin concentrate. If put for a day or two on this diet, *e.g.*, the cocoa-plus-vitamin diet in fig. 14 ("re-education," fifth period of experiment), his condition improves immediately, and when he is once again offered the same choice he no longer selects the "bovril" diet as formerly, but the "cocoa" diet instead. In this way the vitamin concentrate may be transferred from any diet to another, and the rat so deceived, but he can be re-educated, by the method just indicated. Different rats when offered the identical choice will each select a different diet, whichever one contains the distinctive flavouring associated with their own cure.

Part IV.

Some further experiments may now be referred to in support of our conclusion that it is the association between the character of the food in question and an experience of the immediate response which it has evoked which enables the depleted rat to disciminate in favour of the beneficial food.

Experiment 15.—*Immediate Nature of the Response with Provision of Vitamin B.*

This is well illustrated in fig. 15. On each instance when the rat takes a sufficient amount of the vitamin B containing food (shown by asterisk) his weight curve shows a closely parallel response, and if on any occasion the vitamin-free foods be sampled instead there is a corresponding loss in weight, failure of appetite, etc. A few days' education to the virtues of the vitamin-containing diet suffices to increase his attachment to it. We have already alluded to the speed with which the appetite is restored and the conditions in the digestive tract improved. *Experiment* 16, fig. 16 (from the data of Drury, Harris and Maudsley, 1930), shows that the response to the vitamin-containing diet is almost as dramatic if measured, *e.g.*, by the heart rate: the heart of the vitamin B_1 depleted rat beats at as low a rate as one-half of the normal but

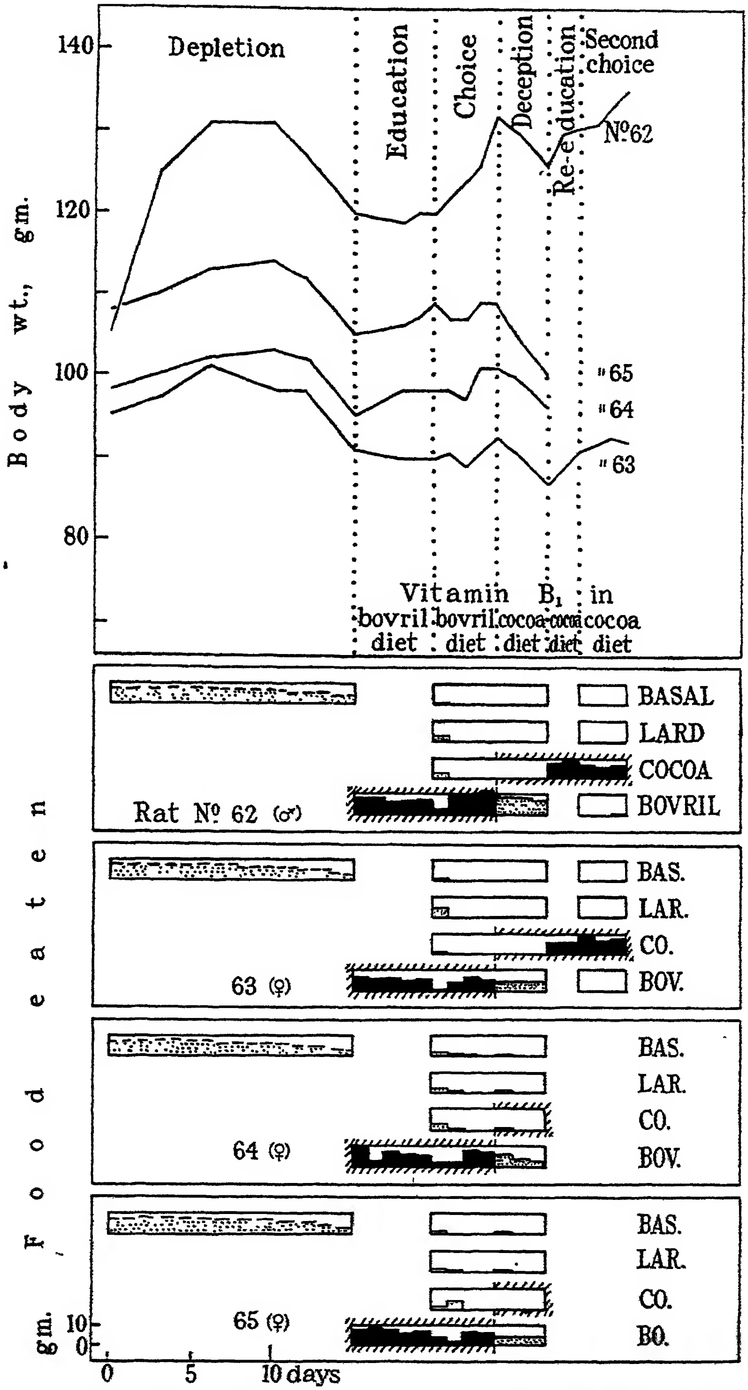


FIG. 14.—Depleted rats, educated to the "bovril" diet, containing vitamin B_1 concentrate, still select it when the vitamin is transferred to the "cocoa" diet. Later, re-education to "cocoa" diet. Shading at edge denotes diet with added vitamin.

some improvement can be detected within less than 1 hour after partaking of the vitamin.

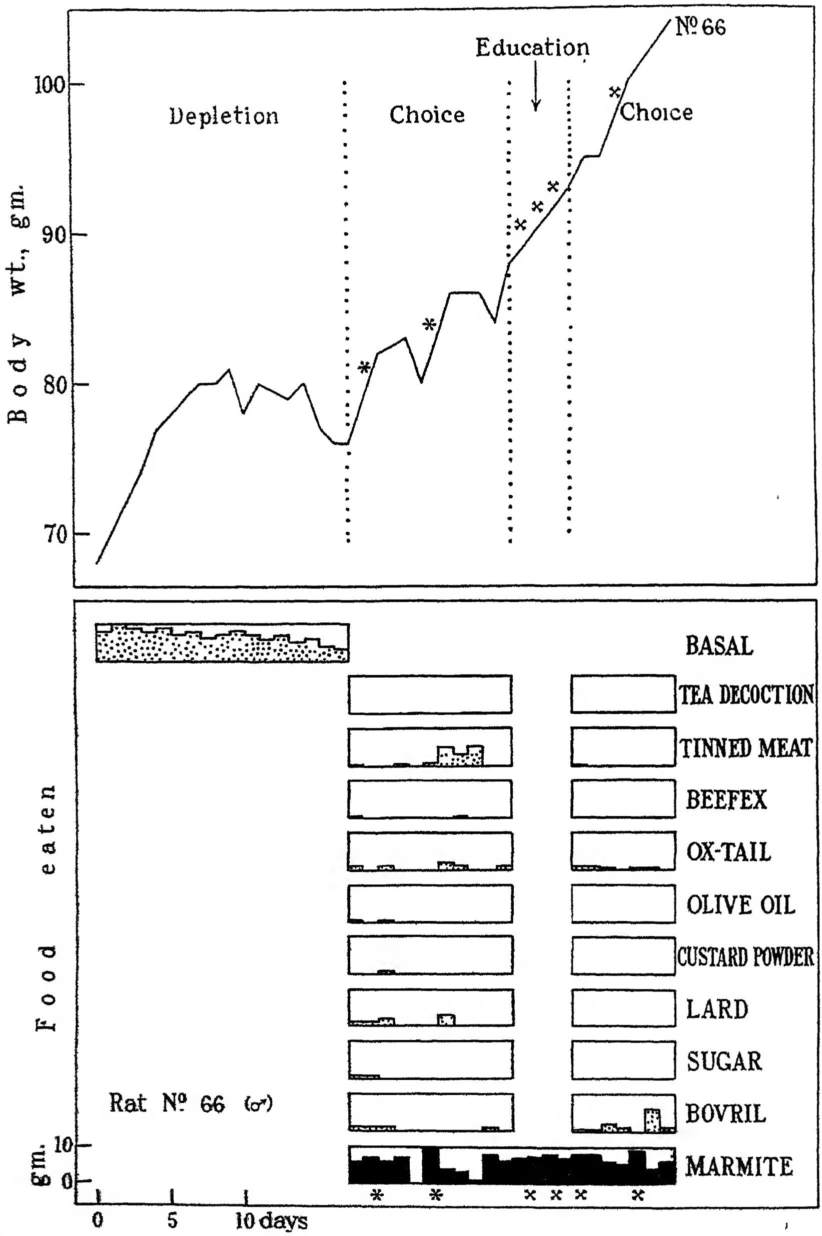


FIG. 15.—Immediate association between the consumption of the vitamin-containing diet (marked * or ✻) and the improvement in condition, as evinced by restoration of growth.

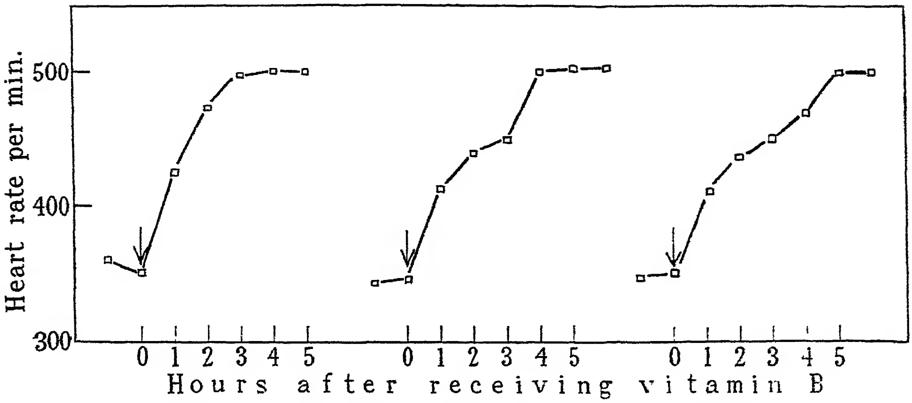


FIG. 16.—Rapidity of cure of bradycardia after administration of vitamin B_1 (marked by arrow).

Experiment 17.—*Failure to Select the Vitamin-containing Diet when the Response which it Evokes is Insufficient.*

Hitherto we have been discussing experiments in which a bare adequacy of vitamin B (or B_1) was allowed. In experiment 17, fig. 17, left hand, rats were offered the choice between a vitamin B free diet and a diet containing only about one-half the bare adequacy of vitamin B_1—in the form of wheat germ extract, *i.e.*, sufficient was present to permit the animal to survive but not enough to give a good growth response; the control curves are shown in fig. 17 at the right hand. Under these circumstances, in the absence of a sufficiently marked reaction resulting from the consumption of the vitamin-containing diet, the animal failed to discriminate satisfactorily between it and the vitamin-free diet.

The converse effect when more than the bare adequacy of vitamin is provided—percentage of marmite in diet was here 7·5, instead of 5, as in previously described experiments—is shown in the last section of fig. 17, left hand. At first the depleted animal chooses the vitamin-containing diet almost exclusively, but later, as he is satisfying his full needs for the vitamin and getting no better response by restricting himself to the vitamin-containing diet only, he begins to take increasing amounts of the vitamin-free diet also.

Experiment 18.—*Negative Results with Dietary Essentials Other than Vitamin B, Provision of which does not Produce so Immediate a Response.*

Experiments on similar lines have been carried out to test whether a rat depleted of, and therefore in urgent need of, some other dietary essential—

such as vitamin A, or vitamin D, or protein—shows any ability to select a diet containing an adequancy of the constituent in question, in preference to other diets deficient in it. In contrast with the results for vitamin B, these experiments have yielded consistently negative results. It therefore seems a likely conclusion, in keeping with what has already been deduced, that the reason why the rat was unable in these cases to select the beneficial diets, was because they

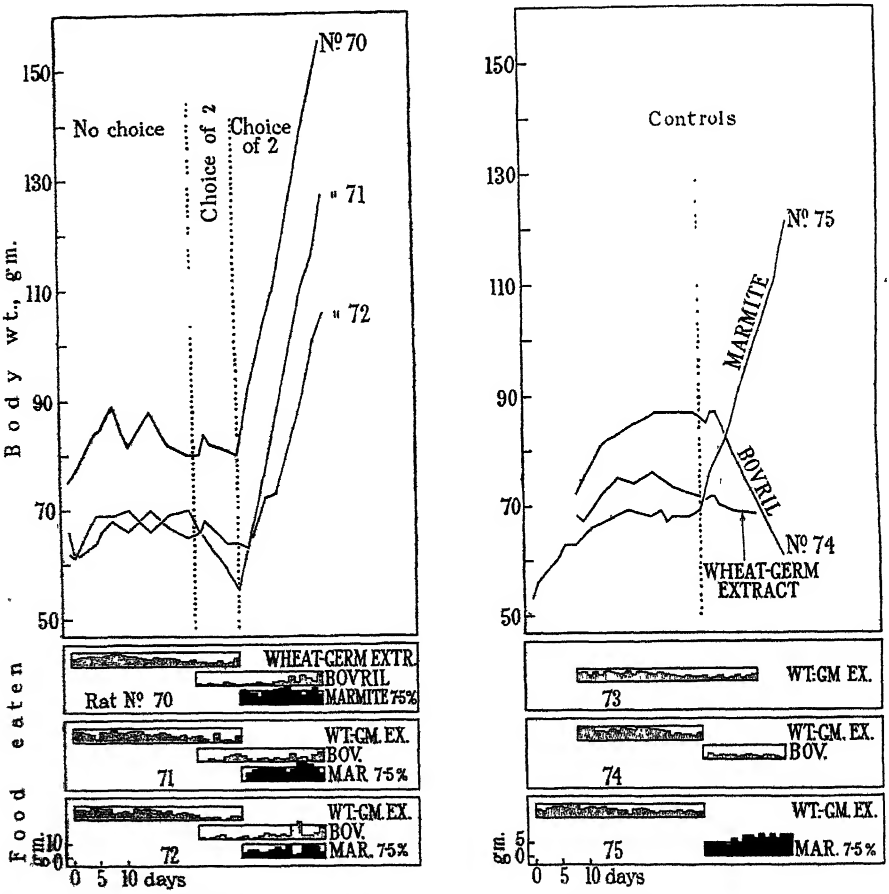


FIG. 17.—Less exclusive choice when the diet contains either (1) less, or (2) more, than the bare adequacy of the vitamin, and the response is less marked. Controls on right.

failed to evoke the stimulus of a sufficiently immediate and marked remedial effect. An account of these negative results together with a discussion of the possibility of securing, even here, some degree of choice, by a specially devised system of "education," will be given in a later paper. Attacking the problem from the opposite side, we have had some success in showing that diets

constituted so as to produce some immediate distasteful reaction, will generally be rejected by the rat. Thus distinctively characterized diets containing a sufficient excess of vitamin D to produce severe hypervitaminosis will frequently be refused by experimental animals and alternative harmless diets free of the excess selected instead (Harris and Moore, 1928). This effect, however, was far less striking and less constant than the choice for vitamin B by depleted rats. The latter without doubt is a special case depending on the exceptional rapidity of the response. Further work is in progress in connection with "salt craving." The fact that an animal which has been deprived of common salt experiences an immediate benefit once it is provided with it, suggests that the desire for salt may have a similar basis to that for vitamin B.

Additional Comments.

The view generally accepted by psychologists at the present time is that the choice of diet by animals is a matter of inherited instinct. The flavours associated with certain foods are supposed to be "palatable" and others "unpalatable." Perhaps the most novel feature therefore in our findings is the conclusion that the ability to choose foods containing the vitamin, in the case of the vitamin B depleted rat, is due not to any inherited instinct for the right food but rather to a previous experience of the beneficial results which follow its consumption. As we have seen, this previous experience may at times lead the animal into error, causing it to choose the wrong diet, and deceiving it into associating its recovery not with the beneficial ingredient actually responsible but with some other feature of the diet acting upon its special senses. In place of a "little of what you fancy does you good" one might write "you get to fancy a little of what did you good *promptly*."

We think it likely that future work will show that "*experience*," as contrasted with the "*instinct*" to which attention has hitherto been so largely directed, plays an important part in determining human appetite. Most obviously perhaps, the taste for alcoholic beverages might be considered to originate not so much in any inherited partiality for their flavour, but more likely in an association which exists in the mind of the addict between the consumption of the beverage in question and the euphoria to which it gave rise in previous experience. Desire for coffee and tea and other stimulating articles of diet, we would suggest, likewise cannot easily be explained on the lines of the classical "palatability" theory, but is again probably an acquired taste, depending originally on a recollection of the "refreshed," or "warmed," or "awakened"

sensations following their use on an earlier occasion. The association without doubt is often subconscious rather than fully reasoned, and tends to pass into an automatic habit. That the craving of a thirsty individual for fluid or of a chilled one for hot food rests on the same basis seems almost self-apparent. We would extend the argument so far as to suggest that the feeling of well-being engendered in a hungry man by the consumption of, say, a juicy steak or the satisfaction following a hearty dinner, must surely play a large part in determining his appetite on future occasions. It seems reasonable, indeed, to suggest that the very desire for food itself (as apart from the choice between different articles of diet) has an experiential no less than an instinctive side.

Of practical interest in connection with our experiments is the method of baby feeding which was originally advocated in America and appears to have won some measure of support (Davis, 1928; Scurfield, 1929), *i.e.*, of allowing infants at weaning to determine their own dietaries by a system of voluntary selection—both in respect of constituents and of quantity. We believe that the idea underlying this proposal is fallacious, and that choice of diet can no more be left to instinct than can the other activities of maternity and child welfare, where until recently it was widely held that "Nature," or instinct, was a sufficiently sure guide. Judging from our experiments, as well as on general common sense grounds, it is hard to see how an infant can be expected to discover the need for a dietary constituent which although it may be without any *immediate* obvious effect may yet, by its continued cumulative action, be absolutely essential for its future well-being. Indeed, as Mottram (1929) has emphasized, children become attached to particular articles of diet largely as a result of usage; and—reversing the old-fashioned procedure—a modern baby, brought up to relish cod liver oil, can only be persuaded to accept jam when it is offered for the first time by imparting to it a sufficiently "attractive" cod liver oily disguise. While our experiments suggest that the need for certain dietary essentials can sometimes be discovered subconsciously by the individual from experience, there seems little doubt that, speaking as a whole, the right choice of diet cannot be left to instinct but is an art that has to be taught.

Summary.

Part I.—A rat depleted of vitamin B (or B_1) and then offered the choice of two diets, one devoid of the vitamin, and the other containing not more than a bare adequacy of it—and in some distinctive form, such as marmite or yeast or wheat germ—will almost invariably select the latter diet. On the other hand, a rat not so depleted will eat the two diets indiscriminately, until it begins to suffer from

the vitamin deficiency, when it will also begin to exhibit the preference. The depleted rat will prefer to continue indefinitely on the same monotonous diet, containing the bare adequacy of the vitamin, in preference to new diets devoid of it. The latter when first offered are "tested" in negligible quantities only. (Should the vitamin-containing diet placed before the depleted rat contain *more* than the bare adequacy of the vitamin, he may cease, after a time, to restrict himself to it exclusively.)

PART II.—If the number of foods placed before the depleted rat is made sufficiently large, the animal may be unable to "discover" the vitamin-containing diet. If at this stage the vitamin-containing diet be fed alone for a few days ("education" period), the experience thus gained now enables him to make the correct selection when once again confronted with the same large choice. Occasionally an animal succeeds in discovering unaided the vitamin-containing diet from among even a very large choice, and once he has succeeded in so doing, he will continue to eat it indefinitely.

PART III.—If the vitamin is incorporated not as a natural source but as a minute addition of a highly potent concentrate the rat may be unable to differentiate between the vitamin-containing and the vitamin-deficient diets—which can be made identical apart from the presence in the one of the vitamin in the active and in the other in the inactivated condition. But if a distinctive character is given to the vitamin-containing diet by the addition of some inert material, *e.g.*, bovril, the choice is readily made. If now the vitamin concentrate is transferred to another diet, *e.g.*, cocoa flavoured, the rat is "deceived," and continues for a time to eat the diet formerly containing the vitamin, *i.e.*, bovril flavoured. But he can be "re-educated" to choose whichever diet now contains the vitamin, provided it possesses a distinctive characteristic.

PART IV.—*From the above and other evidence it is concluded that the ability of the Vitamin B depleted rat to discriminate between diets containing the vitamin and those deficient in it depends not on vague instinct but on an association between the distinctive character of the diet (smell, taste or appearance) and an experience of the prompt beneficial effects,* e.g., *on appetite, alimentary tone, etc., which follow immediately on its consumption. With certain other dietary essentials, where the benefit is less immediately apparent, the rat is unable to distinguish between the deficient and the adequate diets.*

The factor of "experience" is believed to be of wide significance in determining dietary preferences in general.

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Certain Functions of the Midbrain in Pigeons.

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(From University College, London.)

(Communicated by G. Elliot Smith, F.R.S.—Received September 15, 1932.)

I.—In 1928, in the course of our investigations on the sympathetic system, we found that electrical and mechanical stimulation of the pigeon's brain revealed some striking peculiarities.

During that time, a communication to the Physiological Society of the results obtained by Professor W. H. Wilson* of Cairo (presented by Dr. H. A. Harris), which have not yet been published, made us acquainted with phenomena exhibited by the lizards *Varanus* and *Uromastix*, which are identical with those seen by us in pigeons.

Dr. Wilson found that in the cortex of the colliculus in lizards there is a kind of sensory-motor area, with definite localization for various movements and postures of the body, roughly comparable with those determined by the precentral convolution in the cerebral hemispheres of mammals. In fact, he obtained in *Varanus* and in *Uromastix* by electrical stimulation definite areas for flexion of the head on the neck; abduction of the head; extension of the head and neck; rotation of the head; movements of the tail, of the fore-limb and the hind-limb, and contraction of the sterno-mastoid. All these movements were produced by stimulating certain definite points of the cortex of the colliculi and the effects were displayed on the side stimulated. Excepting the latter characteristic, it seems that the functions analogous to those of motor cortex of mammals are performed in the midbrain of lizards by the cortex of the colliculi.

We found exactly the same thing in the pigeon. Briefly, the localizations which we noted, and repeatedly confirmed, on the pigeon's colliculi are as follows :—

(*a*) Only a limited area on the surface of each colliculus is excitable. This area is indicated in figures 1 to 4 by cross-hatching, and it extends from the anterior (inferior) to the posterior (superior) pole of the colliculus, which occupies the medial position.

* Quoted with the consent of Professor W. H. Wilson.

(*b*) In this area electrical, as well as mechanical, stimulation produces :—

From area (1) movements of the neck (especially extension).
,, (2) ,, neck and wing.
,, (3) ,, wing and leg.
,, (4) ,, tail.

All these movements may be produced by a very weak induction current, or merely by touching the surface of the colliculus. The same current, and even one ten times as strong, did not produce any movement or notable reaction when applied to the rest of the colliculus, to the cerebral hemisphere or to the cerebellum.

(*c*) From the areas noted on figs. 1 and 4 by the numbers 1 and 2, we obtained also movements of the beak (opening and closing) and of the larynx (raising and lowering). These movements are determined, however, by deeper structures in the colliculus and not by the surface as we shall explain later. All these movements are on the same side as the excitation but, when the current is increased in strength, they become bilateral.

(*d*) By plunging the electrode into the depths of the colliculus we found, in addition to the movements already described, a region marked 6 in fig. 1 and *xx* in fig. 4 where excitation produced the whole complex of the movements for the act of "crowing," and actual, persistent "crowing" of the bird.

(*e*) In the same bird (subsequently to obtaining responses by mechanical or electrical stimulation) after complete decerebration, when the bleeding has stopped, the same areas of the colliculus when excited, again yield the same responses, viz., movement of the neck (1), of the wing (2), of the leg (3), of the tail (4), of the beak and larynx (5), and "crowing" (6).

(*f*) For the purpose of localizing these areas with greater precision we exposed the colliculi on both sides and, with a red-hot spatula, quickly destroyed the surface of the colliculi by superficial burning all round. In the same sequence the characteristic responses to electrical stimulation were suppressed one by one. After this operation, electrical stimulation still caused movements of the beak and larynx (5 on fig. 1 and *x* on fig. 4).

(*g*) In a second operation in the same bird (with decorticated colliculi) the whole mass of the colliculi was destroyed by burning and then no movement of the beak and no crowing were obtainable. The bird became completely blind and "speechless."

(*h*) Mechanical and electrical stimulation, carefully applied in succession to every millimeter of hemispherical cortex and cerebellum gave no motor

response, even when the current was increased to ten times the strength used for obtaining reactions from the midbrain. Needles or electrodes plunged deeply into these structures, or actual transpiercing of the whole mass of the

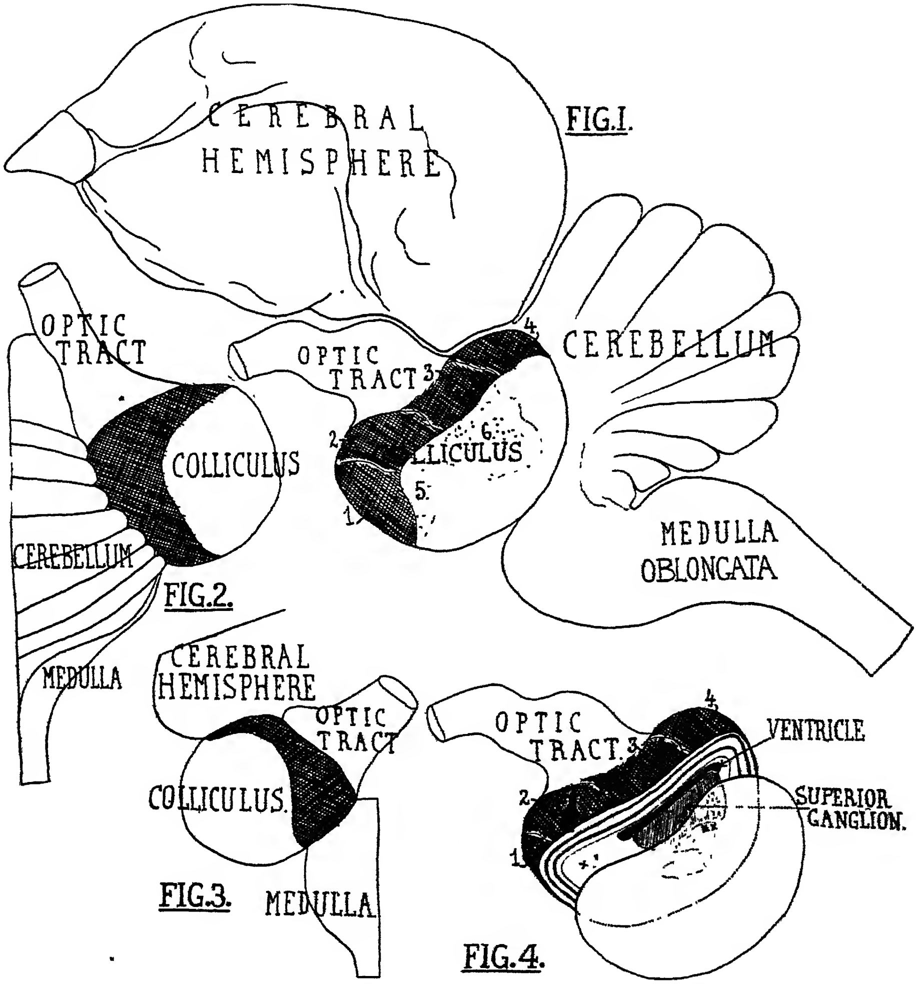

FIG. 1.—Left lateral view of the whole brain of a pigeon. The excitable area of the cortex of the colliculus is indicated by cross-hatching: (1) neck; (2) neck and wing; (3) wing and leg; (4) tail; (5) beak and larynx; (6) crowing ("speech"). FIG. 2.—Dorsal aspect of colliculus, cerebellum and medulla oblongata. The excitable cortex of the colliculus is indicated by cross-hatching. FIG. 3.—Ventral aspect of colliculus, cerebellum and medulla oblongata. The excitable cortex is again indicated. FIG. 4.—Left colliculus cut across at the limit of the excitable cortex. The notations of localizations on the cortex are as in fig. 1. "x" indicates the area where mechanical or electrical stimulation produces movements of the beak and larynx, and "xx" the centre for speech.

hemispheres or cerebellum, produced no motor effect. We were quite unable to obtain from the cerebral cortex such responses as Kalischer (1900) claimed to have evoked in the parrot, or those more recently recorded by Rogers (1922–23).

II.—More recently (1932) one of us, assisted by Dr. A. Abbie (of University College, London) has repeated the above experiments with the same results. The cortical localizations of the colliculi were clearly shown and the more deeply placed centres for crowing, and for movements of the beak and larynx were also constant.

Some new observations were made during this recent series of experiments :—

1. The birds with punctured or partially destroyed colliculi are very excitable, very frightened and have brisk, exaggerated movements.

2. The birds in which both colliculi have been destroyed have dilated, fixed pupils which do not react to light or anæsthetics, but they react to pain and especially to asphyxia.

3. After destruction of both colliculi, the general muscle-tone (skeletal) is decreased, the flapping of the wings is weak, the power of the legs is diminished and the birds tire very rapidly. The co-ordination of movements, however, is perfectly maintained.

4. After every muscular effort the feathers remain ruffled, but at the slightest touch they suddenly become depressed on the body more quickly than in the normal bird. It seems that the motor control of the feathers is disturbed, and that the colliculi play some part in this effect.

5. The birds become absolutely blind when both colliculi are destroyed and when flying they do not avoid obstacles. Auditory memory is retained. Thus when the birds hear the sound of falling wheat in the cage, they listen, become attentive and orient themselves according to the direction of the sound.

6. The memory for performing such complicated acts as picking up corn or drinking water is lost. If placed in a large tin full of wheat, the birds walk around upon the wheat for hours and, although their crops are empty, they do not pick a single grain. When the sound of falling grain is heard, however, the birds become attentive to it and turn towards the direction of the sound which probably reminds them of a pleasant experience. They pay no attention, however, to the wheat under their feet and, if not fed, would die of starvation in the midst of plenty.

7. Some of the complicated motor actions, however, are still retained. The birds clean themselves and preen their feathers as normal birds do.

8. Excitation of the cerebral hemispheres and of the cerebellum, if the correct current is used, never elicits movements of any kind. If the current is greatly increased it may cause various effects by spreading to other parts of the brain.

III.—We do not attempt here a review of the literature relating to this subject.

Many authors have noted movements caused by excitations of the colliculi. In relation to our own observations, we would, however, recall Allen's (1927) observation that there is no area in the cerebral cortex of the rabbit stimulation of which excites movements of the hind limb, such as can still be evoked by electrical stimulation of the superior colliculus. If this is confirmed the rabbit represents a phase in evolution in which the transference of motor functions from the midbrain to the cerebral cortex has only been partially effected.

IV. *Conclusion.*—The scattered facts recorded in the literature, correlated with our experiments, show definitely that in birds there is a real motor cortex with definite localization in the colliculi, but no such thing in the cerebral hemispheres. In the central mass of the same colliculi there is a centre for voice production and for movements of the beak and larynx.

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The Effect of Carbon Dioxide on Bacterial Growth.

By F. P. COYNE, Torry Research Station, Aberdeen.

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The first systematic investigation on the influence of carbon dioxide on micro-organisms was carried out by Fränkel (1889) who corrected and correlated a number of earlier observations. Many papers have since been published on this subject and a number of these are reviewed in the discussion, p. 211.

In a previous paper (Coyne, 1932) preliminary observations regarding the influence of carbon dioxide on the growth of bacteria were reported. The majority of the organisms examined showed marked inhibition of growth in presence of carbon dioxide. These results, which were obtained by incubation of cultures at 14°–18° C. for 4 days, have now been extended and the effect of various proportions of carbon dioxide (5 per cent. to 100 per cent.) at different temperatures has been investigated over considerable periods. In addition, the statement that the effect cannot be attributed solely to the p_H of the medium has been amply confirmed by further experiments.

Technique.

Only by such a roughly quantitative method as is described below could results be obtained on a large number of cultures within a reasonable time and it was considered more important, initially at least, to make a general survey rather than to indulge in an intensive study of a few cultures. The technique used throughout was as follows. Stock cultures were plated out on to horse heart agar* and single colony transfers made into broth† after an incubation period of 2 days at 25° C. Broth cultures were incubated for 2 to 3 days at 25° C., after which one loopful (platinum wire loop, 4 mm. diameter) of culture was inoculated on to each plate to be tested by making seven successive

* *Agar Medium*—Add 1 lb. minced horse heart to 1 litre of 2 per cent. agar at 50°–55° C., and boil 20 minutes. Filter hot through sieve and pass filtrate through residual pad twice. Add 1 per cent. peptone and 0·5 per cent. NaCl and boil 10 minutes. Adjust p_H to 7·8 and allow to cool in steamer overnight. Cut off sediment and sterilize remainder.

† *Broth Medium*—Autoclave 1 lb. minced horse heart in 1 litre H_2O for 20 minutes (120° C.). Filter hot and add 1 per cent. peptone and 0·5 per cent. NaCl. Adjust p_H to 7·8 and filter cold before sterilizing.

strokes across the surface of the agar. Thus each plate received approximately the same inoculum similarly distributed. Examination of plates during incubation was carried out at regular intervals, the amount of growth being marked according to the following scale :—

— no growth visible with hand lens ($\times$ 8).
$\pm$ faint shadow on first line of inoculation, naked eye or lens ($\times$ 8)
$+$ distinct growth on first stroke.
$++$ growth on several strokes.
$+++$ well developed growth throughout.
$++++$ abundant growth.

A number of inherent difficulties are involved in this method, rendering the exact duplication of results almost impossible. They include :—

(*a*) Variations in the amount of moisture on the surface of plates.
(*b*) Variations in inoculation due to differences in growth of the broth cultures used for inoculating plates.
(*c*) Variations in the type of growth on the plates.

These were largely eliminated for any one experiment by carrying out the complete set at one time under standard conditions, but a repetition of the same experiment usually failed to give an exact replica of the results. The variation occurred generally in the lag period and so affected the carbon dioxide plates more than the air controls. Where this occurred the most favourable growth has been given in the tables so that any bias is against carbon dioxide rather than in its favour.

The requisite proportion of carbon dioxide was obtained by the use of vacuum desiccators. These were partially evacuated and then refilled with carbon dioxide, the amount of which was measured by a mercury pressure gauge. When the desired volume had been added the desiccator tap was opened to air and the final pressure thus brought back to normal. The turbulence produced by the inward rush of air ensured adequate mixing. The accuracy of this method was checked by gas analyses which showd the error to be within 1 per cent. for mixtures containing 5 per cent. to 80 per cent. carbon dioxide. To obtain an atmosphere of 99 per cent. to 100 per cent. carbon dioxide evacuation to 50 mm. pressure and complete refilling with gas was carried out twice and found effective. Previous passage of the gas through water, through solutions of $KMnO_4$ or $NaHCO_3$ or over heated copper to remove impurities, had no effect on the results obtained.

The Influence of Various Concentrations of Carbon Dioxide at Different Temperatures.

Since this investigation was undertaken with special reference to the preservation of fish the cultures used are in the main those isolated by Stewart (1932) from the surface slime and from the intestine of fresh fish. Of 247 cultures isolated from slime 140 correspond in general characters with the genus *Achromobacter* (Bergey). These latter were sub-divided into 15 types of which only one was identical with any previous description. Type 1 was by far the most abundant, comprising 70 per cent. of the total and was divided into four sub-groups on the basis of glucose fermentation and nitrate reduction. Of the others, types 6, 7, 8 and 18 comprised the majority of organisms. A number of strains from each of these types was selected for examination. In Table I the rate of growth in presence of various proportions of carbon dioxide at 0° C., 10° C., and 25° C. is shown in detail for one organism.

At 0° C. growth in air reaches a maximum in 12 days while even after 40 days no growth occurs in presence of 20 per cent. carbon dioxide. Lower concentrations of carbon dioxide give intermediate results. At 10° C. the inhibition is less marked, but in 50 per cent. carbon dioxide, a pronounced lag is noted and no growth occurs in 100 per cent. At the optimum growth temperature of 25° C. the influence of carbon dioxide is further diminished and even in 100 per cent. carbon dioxide growth is visible in 6 days although it is confined to the first line of inoculation.

Similar tables have been constructed for all the 94 organisms examined, but space does not permit of their publication. It has, however, been possible to tabulate the time required in each case to attain an arbitrary degree of growth, namely, distinct growth on the first stroke of inoculation. This method of tabulation suffers from the restriction that it shows nothing of the rate of further development. Frequently the standard growth of + was never exceeded in carbon dioxide while the controls developed to +++, as is shown in Table I with 15 per cent. carbon dioxide at 0° C. and again with 100 per cent. carbon dioxide at 25° C. Nevertheless, the figures shown in Tables II–V suffice for general comparative purposes.

Table II shows the results obtained with those cultures selected as representative of the genus *Achromobacter*.

The most striking observation is the comparatively rapid growth of this genus at 0° C. In air distinct growth is apparent in about 4 to 6 days and the maximum (+++) is attained in 8 to 12 days (not shown on table). This

Table I.—*Achromobacter* No. 1.

Rate of growth in air and in carbon dioxide.

Time, days.	Temperature 0° C.					Temperature 10° C.					Temperature 25° C.				
	Percentage CO_2.					Percentage CO_2.					Percentage CO_2.				
	0.	5.	10.	15.	20.	0.	10.	20.	50.	100.	0.	10.	20.	50.	100.
1						+					+++	++	+	−	−
2						++	+	+	−	−	+++	+++	+++	±	−
3												+++	+++	++	−
4	±					+++	++	+	−	−				+++	−
6	++	+	+	±	−	+++	+++	+++	±	−				+++	±
8	++	+	+	±	−		+++	+++	+	−					+
10									+	−					+
12	+++	++	+	±	−				++	−					+
14									++	−					+
16	+++	+++	++	+	−				++	−					+
18		+++	++	+	−				+++	−					+
20			++	+	−				+++	−					
24			+++	+	−					−					
28			+++	+	−					−					
40				+	−					−					

Species.	Culture number.	Temperature 0° C. Percentage CO_2.					Temperature 10° C. Percentage CO_2.					Temperature 25° C. Percentage CO_2.				
		0.	5.	10.	15.	20.	0.	10.	20.	50.	100.	0.	10.	20.	50.	100.
Achromobacter, type 1A …………	1	5	6	6	16	>28	1	2	2	12	>40	<1	<1	1	3	9
	110	8	14	28	>40	>40	1	1	2	>40	>40	<1	<1	1	1	>15
	131	6	16	>40	>40	>40	<1	1	2	28	>40	<1	<1	2	3	>15
	180	6	16	>40	>40	>40	1	1	2	28	>40	<1	<1	2	2	>15
	190	6	14	>40	>40	>40	<1	1	2	14	>40	<1	<1	1	2	>15
	201	8	14	>40	>40	>40	1	2	2	12	>40	<1	<1	1	>15	>15
	280	4	6	6	6	14	<1	1	2	8	>40	<1	<1	1	2	>15
	305	6	6	40	>40	>40	1	1	3	20	>40	<1	<1	1	3	3
Achromobacter, type 1B …………	102	4	6	14	14	>40	<1	1	2	22	>40	<1	<1	<1	>15	>15
	127	8	14	14	>40	>40	<1	1	2	>40	>40	<1	<1	1	5	>15
	157	4	8	8	14	40	1	1	2	12	>40	<1	<1	1	3	>15
Achromobacter, type 1C …………	15	5	6	14	16	>40	<1	1	3	16	>40	<1	<1	1	3	>15
	50	8	14	32	>40	>40	1	2	4	>40	>40	<1	2	2	3	>15
	75	6	8	14	14	36	1	1	2	8	>40	<1	<1	<1	3	6
	100	4	6	6	8	>40	<1	<1	2	12	>40	<1	<1	<1	3	>15
	116	6	8	14	14	>40	1	1	2	6	>40	<1	<1	<1	6	>15
Achromobacter, type 1D …………	23	5	6	6	14	>40	<1	1	2	10	>40	<1	1	1	4	>15
	63	4	6	6	>40	>40	<1	1	4	24	>40	<1	1	1	3	>15
	142	6	8	10	40	>40	1	2	2	10	>40	<1	1	2	3	>15
Achromobacter, type 6………………	60	5	6	7	8	>28	2	2	3	>40	>40	<1	<1	1	>15	>15
	165	6	6	14	36	>40	<1	1	2	>40	>40	<1	<1	4	>15	>15
Achromobacter, type 7…. …………	39	4	7	8	8	14	<1	1	2	5	20	<1	<1	<1	2	3
	146	6	6	8	12	16	<1	<1	2	6	>40	<1	<1	<1	3	>15
	152	4	6	8	12	>16	1	1	2	6	16	<1	<1	<1	1	2
	277	6	6	6	8	14	<1	1	1	5	20	<1	<1	1	1	2
Achromobacter, type 8………………	147	4	6	6	6	16	<1	1	1	6	>40	<1	<1	<1	2	8
	160	4	6	16	>16	>16	1	1	3	28	>40	<1	<1	<1	3	>15
Achromobacter, type 18 …………	328	>40					1	1	1	4	6	<1	1	1	2	2
	340	>40					<1	1	1	4	6	<1	1	1	2	2

finding is of considerable importance when correlated with the fact that the genus *Achromobacter* preponderates so largely in fish slime and with the conclusions of Lumley, Piqué and Reay (1929) that the deterioration of fresh fish on board trawlers is mainly due to bacteria and that 10 to 12 days is the maximum period during which fish can be kept in reasonably fresh condition when packed in ice.

At all three temperatures a distinct lag occurs in presence of carbon dioxide, increasing with the concentration of this gas. The effect is more pronounced at low temperatures. The two organisms representing type 18 differ from the others in that they fail to grow at 0° C. in air and at higher temperatures are less affected by carbon dioxide than the majority. These cultures were isolated from the intestine of haddock while the others all originated in slime on the surface of fish. Forty-six cultures were isolated from intestinal contents by Stewart (1932) who again found the genus *Achromobacter* to predominate largely.

Amongst other bacteria found in fish slime the genus *Micrococcus* is numerically next in importance, forty cultures having been isolated. Twenty-seven cultures of *Flavobacter* were obtained from slime and one from the intestine. Most samples of intestinal contents examined by Stewart contained a few aerobic spore-bearing organisms, although these were never found in slime. Their characteristics corresponded with those of known members of the genus *Bacillus*. From slime were also isolated five cultures of the genus *Pseudomonas* all of which agreed in characteristics with known species. In Table III are given the results of an examination of representative cultures from these genera.

In general the results are similar to those obtained with the genus *Achromobacter*.

Investigations were extended to bacteria isolated by Stewart (1931) from decomposing fish muscle. Of 84 cultures 34 belong to the genus *Achromobacter* which is again in a majority. 19 cultures of *Pseudomonas*, 8 of *Flavobacter* and 7 of *Micrococcus* were also isolated. These all corresponded with strains found in slime and intestinal contents and were therefore not subjected to further examination. No spore-bearing organisms were obtained, but a few culures of *Aerobacter* and of *Proteus* were isolated.

Four cultures of *Aerobacter* had occurred in fish slime and one in intestinal contents, but no member of the genus *Proteus* had been obtained previously. Preliminary experiments showed that neither of these two genera is affected by carbon dioxide to the same extent as the other bacteria examined. It was

Table III.—Time in days to attain standard growth of +.

Species.	Culture number.	Temperature 0 °C.					Temperature 10° C.					Temperature 25° C.				
		Percentage CO_2.					Percentage CO_2.					Percentage CO_2.				
		0.	5.	10.	15.	20.	0.	10.	20.	50.	100.	0.	10.	20.	50.	100.
Flavobacter, type 28	51	>40					4	6	8	8	>40	1	2	2	3	5
,, ,, 29	88	>40					4	6	10	>40	>40	2	2	2	3	15
,, ,, 30	9	12	>28	>28	>28	>28	3	3	6	8	>40	<1	1	2	6	>15
,, ,, 32	2	12	16	16	>28	>28	2	3	4	4	8	<1	<1	<1	1	2
Micrococcus, type 20	73	18	18	24	>40	>40	3	4	6	8	16	1	1	1	3	9
,, ,, 22	89	>40					4	8	16	>40	>40	1	2	2	4	>15
,, ,, 24	111	>40					2	6	6	8	12	<1	<1	<1	<1	1
,, ,, 25	49	>40					4	4	12	16	>40	<1	<1	<1	<1	1
Bacillus sublanatus	345	>40					2	4	10	>40	>40	<1	1	1	3	>15
,, *cereus*	352	>40					2	4	12	>40	>40	<1	<1	<1	1	2
,, *mesentericus*	388	>40					3	5	10	>40	>40	<1	<1	<1	<1	2
,, *mycoides*	389	>40					3	6	12	>40	>40	<1	<1	<1	1	2
Pseudomonas fluorescens	19	6	6	12	16	18	1	1	2	6	>40	<1	<1	<1	1	4
,, *viscosa*	26	6	10	12	16	20	2	3	4	6	16	<1	<1	<1	2	6
,, *convexa*	38	4	8	12	16	24	<1	2	4	8	>40	<1	<1	1	2	4

decided, therefore, to test all the strains isolated by Stewart since, although these organisms do not appear to be part of the normal flora of fish, they are of importance owing to their widespread occurrence and to the ease with which they may contaminate fish during handling. The results are shown in Table IV.

The genus *Aerobacter* is represented here by two species *A. cloacæ* and *A. aerogenes* of which the former is sub-divided into two strains differentiated by gelatin liquefaction which occurs only with sub-group A. The genus *Proteus* is represented by one species only, *P. vulgaris,* but while two of the strains are typical (A) the other four (B) fail to liquefy gelatin.

Owing to the resistance of these organisms it was not considered of value to test the effect of less than 50 per cent. carbon dioxide which, however, has a pronounced effect at 0° C. and at 10° C. At 25° C. there is no appreciable difference on the standard of + growth even in 100 per cent. carbon dioxide. The time required to develop to the normal growth of +++ has therefore been added to the table and a distinct lag is then noted in carbon dioxide.

By the courtesy of Professor J. Cruickshank, Department of Bacteriology, University of Aberdeen, an additional set of stock cultures was made available for investigation. These, together with a few cultures purchased from the National Collection of Type Cultures, have also been examined, with the results shown in Table V.

Since the optimal temperature of most of these organisms is 37° C. the lowest temperature used was 10° C. at which many failed to develop. Of those which showed growth at 10° C. considerable inhibition occurred in presence of carbon dioxide. At 25° C. and at 37° C. this was much less marked and the corresponding time to develop to +++ at 37° C. has been appended. Briefly, carbon dioxide has the greatest effect on the genera *Mycobacterium* and *Bacillus*. Most of the other cultures and in particular those of the genus *Bacterium*, resemble the *Aerobacter* and *Proteus* cultures previously examined. An example of extreme variation within a genus is afforded by *C. diphtheriæ* and *C. hofmannii*. It is notable that of the four members of the genus *Bacillus* examined here only one grew at 10° C. and that very slowly, while the four cultures of this genus which had been isolated from fish intestine all grew well at 10° C.

The influence of carbon dioxide on the growth of strict anaerobes was also investigated, using the agar medium previously described (p. 196). For these experiments the metal anaerobic jar of Wilson (1928) was used. The air was exhausted, hydrogen was passed in and traces of oxygen removed by palladium

Table IV.

Species.	Culture number.	Time in days for standard growth of +.									Time in days for standard growth of +++.		
		Temperature 0° C.			Temperature 10° C.			Temperature 25° C.			Temperature 25° C.		
		Percentage CO_2.			Percentage CO_2.			Percentage CO_2.			Percentage CO_2.		
		0.	50.	100.	0.	50.	100.	0.	50.	100.	0.	50.	100.
Aerobacter cloacæ A.	183	12	>40	>40	1	2	4	<1	<1	<1	1	2	3
	242	12	>40	>40	1	2	4	<1	<1	<1	1	2	3
	243	7	>40	>40	1	2	6	<1	<1	<1	1	2	3
	245	12	>40	>40	1	2	6	<1	<1	<1	1	2	3
	248	12	>40	>40	1	2	4	<1	<1	<1	1	2	3
	263	12	>40	>40	1	2	4	<1	<1	<1	1	2	3
Aerobacter cloacæ B.	41	6	20	36	1	2	4	<1	<1	1	1	2	3
	395	7	20	>40	1	2	4	<1	<1	<1	1	2	3
	396	6	20	>40	1	2	4	<1	<1	<1	1	2	3
	397	6	20	>40	1	2	4	<1	<1	<1	1	2	3
Aerobacter aerogenes	294	>40			1	2	4	<1	<1	<1	1	2	3
Proteus vulgaris A.	270	>40			1	2	6	<1	<1	<1	1	2	3
	272	>40			2	2	4	<1	<1	<1	1	2	3
Proteus vulgaris B.	253	>40			1	2	6	<1	<1	<1	1	2	3
	260	>40			1	2	6	<1	<1	<1	1	2	3
	262	>40			2	2	10	<1	<1	<1	1	2	3
	264	>40			2	2	8	<1	<1	<1	1	2	3

after which the hydrogen was replaced by oxygen-free carbon dioxide. Table VI shows the rate of growth in carbon dioxide and in hydrogen at 37° C. Again a very marked inhibition is observed, three of the five cultures failing completely to show any sign of growth after 15 days in presence of carbon dioxide (100 per cent.).

Comparison between the Effect of Carbon Dioxide and the Effect of Hydrogen Ion Concentration.

It had previously been observed (Coyne, 1932) that by passing carbon dioxide into melted agar medium the p_H is reduced from 7·4 to 6·2, but that growth of bacteria at p_H 6·2 in air is much better than in the ordinary medium (initial p_H 7·4) in presence of carbon dioxide. This point has now been investigated in more detail. Standard agar plates of p_H 7·0, 6·8, 6·4, 6·2, 6·0, 5·8 and 5·6 were prepared, each containing 20 c.c. of previously adjusted medium plus the required indicator. For the range of p_H 7·0–6·4 bromthymol blue (0·8 c.c.) was used and for the range 6·6–5·6 chlorphenol red (0·4 c.c.) was used. Test plates each containing 20 c.c. of agar medium of p_H 7·0 plus indicator were incubated in carbon dioxide at 25° C. and at intervals were compared with the standard plates. A similar experiment was carried out with agar medium of initial p_H 8·0. Alterations in p_H were very clearly shown. The results are given in Table VII.

This gives an accurate picture of the changes which occurred in the p_H of the medium during the previous experiments. Identical results were obtained on repeating these tests at room temperature and at 0° C. so the p_H shift is approximately constant within this range of temperature. The lowest value attained is 5·6 and equilibrium is reached in a very short time. The difference between an initial p_H of 8 and one of 7 is not marked but may be significant when the lower limit of p_H tolerance lies about 5·8–5·6 which happens with some organisms. The initial p_H of the agar media used in the previous growth experiments was kept within the limits of 7·2–7·8.

The limiting p_H value for growth, under the conditions adopted, of a selection of organisms susceptible to carbon dioxide was next investigated. Two of the cultures (Nos. 50 and 100) failed to grow at p_H 5·6, and in these cases inhibition by carbon dioxide may be attributed to the effect of p_H. Three (Nos. 110, 131 and 180) failed to grow at p_H 5·4 but grew fairly well at p_H 5·6, while the remaining five showed good growth at p_H 5·4. Comparison of the rate of growth of these five in air at p_H 5·4 and in 50 per cent. carbon dioxide (p_H 5·8)

Table V.

Culture.	Source.*	Time in days for standard growth of +.									Time in days for standard growth of +++.		
		Temperature 10° C.			Temperature 25° C.			Temperature 37° C.			Temperature 37° C.		
		Percentage CO_2.			Percentage CO_2.			Percentage CO_2.			Percentage CO_2.		
		0.	50.	100.	0.	50.	100.	0.	50.	100.	0.	50.	100.
Staphylococcus aureus A.	B.	6	>40	>40	<1	<1	2	<1	<1	1	1	1	2
,, ,, *B.*	B.	4	>40	>40	<1	<1	1	<1	<1	<1	1	1	2
Streptococcus hæmolyticus	B.	>40			1	4	4	1	1	2	2	2	7
,, *lactis*	B.	12	14	>40	<1	1	2	<1	<1	1	1	4	5
Neisseria catarrhalis	N., No. 1339	>40			3	>14	>14	1	>7	>7	2		
Mycobacterium phlei	N., No. 525	>40			2	>14	>14	1	2	6	3	5	7
,, *smegmatis*	B.	>40			1	>14	>14	1	2	6	3	4	7
,, *berolinensis* (Rabinowitsch)	B.	16	>40	>40	2	4	>14	1	2	6	3	4	7
,, *stercusis*	N., No. 526	8	>40	>40	1	2	>14	<1	1	3	2	3	6
Corynebacterium diphtheriæ	B.	>40			<1	< 1	1	<1	1	1	2	2	3
,, *hofmannii*	B.	>40			1	>14	>14	1	>7	>7	3		
Vibrio choleræ	N., No. 1633	>40			<1	3	6	<1	<1	1	1	5	7
Spirillum rubrum	N., No. 953	>40			6	>14	>14	>7					
Proteus X 19	B.	4	>40	>40	<1	1	2	<1	<1	2	1	2	5

Bacterium coli communis	B.	4	6	16	<1	<1	1	<1	<1	1	1	1	3
,, *typhosum*	B.	16	>40	>40	<1	<1	2	<1	<1	1	1	1	3
,, *paratyphosum B.*	B.	4	6	16	<1	<1	1	<1	<1	1	1	1	3
,, *gallinarum*	B.	>40			<1	<1	1	<1	<1	1	1	1	5
,, *pullorum*	B.	20	>40	>40	1	2	2	<1	<1	1	2	5	6
,, *fœcalis alkaligenes*	B.	4	12	>40	<1	1	5	<1	<1	1	1	2	5
,, *shigæ*	B.	>40			1	2	3	<1	<1	1	1	1	6
,, *flexneri* V.	B.	36	>40	>40	<1	1	2	<1	<1	1	1	1	7
,, *pneumoniæ friedländer*	N., No. 204	6	12	>40	<1	<1	1	<1	<1	1	1	1	5
,, *aertrycke*	N., No. 115	4	4	8	<1	<1	1	<1	<1	<1	1	1	2
,, ,,	N., No. 3047	5	6	12	<1	<1	1	<1	<1	<1	1	1	2
,, ,,	N., No. 3048	4	6	12	<1	<1	1	<1	<1	<1	1	1	2
,, *enteritidis gærtner*	N., No. 127	8	10	12	<1	<1	1	<1	<1	<1	1	1	2
,, ,,	N., No. 3045	5	6	6	<1	<1	1	<1	<1	<1	1	1	2
,, ,,	N., No. 3046	4	5	6	<1	<1	1	<1	<1	<1	1	1	2
Bacillus anthracis	B.	>40			<1	1	3	<1	1	1	1	2	3
,, *mesentericus viscosus*	B.	>40			<1	1	5	<1	<1	1	1	1	7
,, ,, *vulgatus*	B.	8	>40	>40	<1	<1	5	<1	<1	1	1	1	6
,, ,, *ruber*	B.	>40			<1	<1	2	<1	<1	1	1	1	5

* B. = Bacteriology Department, Marischal College, University of Aberdeen. N. = National Collection of Type Cultures.

Table VI.

Culture.	Source.*	3 days.		5 days.		15 days.	
		H_2.	CO_2.	H_2.	CO_2.	H_2.	CO_2.
Clostridium sporogenes	N. No. 532	+++	–	+++	–	+++	–
,, *septique*	N. No. 548	++	–	++	–	+++	+
,, *welchii*	N. No. 273	++	–	++	–	+++	–
,, *tetani*	B.	++	–	+++	–	+++	–
,, *botulinum*	N. No. 2012	++	–	+++	–	+++	+

* B. = Bacteriology Department. Marischal College, University of Aberdeen. N. = National Collection of Type Cultures.

Table VII.

Time in hours.	p_H in carbon dioxide concentration of—			
	10 per cent.	20 per cent.	50 per cent.	100 per cent.
0	7·0	7·0	7·0	7·0
3	6·4	6·2	5·8	5·8
6	6·2	6·0	5·8	5·6
24	6·2	6·0	5·8	5·6
48	6·2	6·0	5·8	5·6
0	8·0	8·0	8·0	8·0
3	6·8	6·2	6·0	5·9
6	6·2	6·2	6·0	5·8
24	6·3	6·2	6·0	5·8
48	6·2	6·2	6·0	5·8

showed that the inhibition produced by carbon dioxide is greater than is the effect of p_H alone. The data are given in Table VIII.

To obtain a direct comparison between the effect of carbon dioxide and of p_H the growth of the above ten organisms at 25° C. was measured, using the standard technique, under the following conditions:—

(*a*) In air .... initial p_H 7·6.

(*b*) In air .... initial p_H 5·8.

(*c*) In 50 per cent. N_2 .... initial p_H 5·8.

(*d*) In 50 per cent. CO_2 .... initial p_H 7·6.

(*e*) In 50 per cent. CO_2 + 10 per cent. O_2, initial p_H 7·6.

Table VIII.

Culture.	—	1 day.	2 days.	3 days.
Achromobacter 147	Air, p_H 5·4 50 per cent. CO_2	++ +	+++ ++	+++ ++
Pseudomonas 19	Air, p_H 5·4 50 per cent. CO_2	++ +	+++ ++	+++ +++
Pseudomonas 26	Air, p_H 5·4 50 per cent. CO_2	+ ±	+++ +	+++ ++
Bacillus 345	Air, p_H 5·4 50 per cent. CO_2	+ −	++ +	+++ +
Bacillus 389	Air, p_H 5·4 50 per cent. CO_2	+++ +	+++ +++	+++ +++

The results are given in Table IX.

The control set in air at p_H 7·6 all grew well in 24 hours and reached a maximum in 2 days. No difference can be noted between the sets in air at p_H 5·8 and in nitrogen at p_H 5·8. Both are only a little slower in growth than the control at p_H 7·6. On the other hand, the growth in both the carbon dioxide sets is very much slower. The variations in oxygen made no appreciable difference. This result cannot be explained on the basis of p_H alone. Even with *Achromobacter* 50, which is most delayed by the p_H change, good growth occurs in 3–4 days while the carbon dioxide plates show complete inhibition. Similar results were obtained by repeating this éxperiment at 10° C. Comparison of Table IX with Tables II and III will show that exact duplication of results in 50 per cent. carbon dioxide is not obtained. As was pointed out previously this is caused by the number of uncontrollable variations involved. As the results in Table IX are of particular importance this experiment was carried out three times, the greatest care being taken to keep conditions standard. Close duplication of results was obtained.

Valley and Rettger (1927) claimed that by using medium buffered with 1 per cent. K_2HPO_4 inhibition of bacterial growth by carbon dioxide can be largely eliminated. In Table X is shown the change in p_H which occurs in such a medium in presence of carbon dioxide.

The alteration is much smaller than in ordinary media and if the effect of carbon dioxide were explicable on the basis of p_H change in the medium one would expect much better growth on such a strongly buffered medium. That this, however, is not so is demonstrated by the results given in Table XI.

Table IX.

Culture.	Air.		Air.			50 per cent. nitrogen.			50 per cent. CO_2.			50 per cent. CO_2 + 10 per cent. O_2.		
	Initial p_H 7·6.		Initial p_H 5·8.			Initial p_H 5·8.			Initial p_H 7·6.			Initial p_H 7·6.		
	1 day.	2 days.	1 day.	2 days.	3 days.	1 day.	2 days.	3 days.	1 day.	2 days.	3 days.	1 day.	2 days.	3 days.
Achromobacter 110	++	+++	++	+++	+++	++	+++	+++	+	+	++	+	+	++
,, 131	++	+++	++	+++	+++	++	+++	+++	±	+	++	±	+	++
,, 180	++	+++	++	++	+++	++	++	+++	+	+	++	+	+	++
,, 50	++	+++	−	±	+	−	±	+	−	−	−	−	−	−
,, 100	+++	+++	++	+++	+++	++	+++	+++	+	++	++	+	++	++
,, 147	+++	++++	+++	++++	++++	+++	++++	++++	+	+++	+++	++	+++	+++
Pseudomonas 19	+++	++++	+++	++++	++++	+++	++++	++++	+	++	+++	+	++	+++
,, 26	+++	+++	++	+++	++++	+++	+++	++++	+	+	++	+	+	++
Bacillus 345	++	+++	++	+++	++++	++	+++	++++	±	+	++	+	++	++
,, 389	+++	++++	++	+++	++++	++	+++	++++	+	++	+++	+	++	+++

Table X.—Effect of Carbon Dioxide on Buffered Medium (1 per cent. K_2HPO_4).

Time in hours.	p_H in carbon dioxide concentrations of:—			
	10 per cent.	20 per cent.	50 per cent.	100 per cent.
0	7·6	7·6	7·6	7·6
3	7·2	7·0	6·8	6·6
6	7·2	7·0	6·6	6·4
24	7·2	7·0	6·6	6·4

Growth in air of a number of cultures on agar medium containing 1 per cent. K_2HPO_4 is not affected by the initial p_H within the range 7·6–6·4. On the other hand, even 50 per cent. carbon dioxide, which reduces the initial p_H from 7·6 to 6·6, has a marked effect on growth. In 100 per cent. carbon dioxide this effect is greatly accentuated. That the effect of carbon dioxide has not, in general, a direct relationship to the p_H of the medium is shown by comparison of Tables IX and XI. In one experiment the initial p_H of the medium is reduced by 50 per cent. carbon dioxide from 7·6 to 5·8 and in the other from 7·6 to 6·6 yet there is no appreciable difference in the degree of growth inhibition.

Discussion.

Whilst the deficiencies of the method used for measuring growth rates of bacteria must not be overlooked, the results obtained are significant. The inhibition produced by carbon dioxide, especially at low temperatures, is of quite a different order from the experimental error involved. Substantiation of the results by the much more accurate method of viable counts is, however, to be desired. Such investigations are at present being carried out* and while the factor of time limits considerably the number of organisms which can be studied the results obtained so far are in complete agreement with those published in this paper.

The resistance of bacteria to carbon dioxide varies greatly in different genera and even in different strains of the same species. In all cases, however, a very definite effect is produced by carbon dioxide at low temperatures, resulting either in a prolonged lag followed by sub-maximal growth or in complete

* Dr. Haines of the Low Temperature Research Station, Cambridge, has taken up the study of bacteria isolated from meat, and Dr. Mary Stewart of the Torry Research Station, Aberdeen, is investigating the organisms isolated from fish. Both are using the viable count method.

Table XI.—Growth in Buffered Medium (1 per cent. K_2HPO_4).

Culture.	Air.		Air.		50 per cent. CO_2.			100 per cent. CO_2.			
	p_H 7·6.		p_H 6·4.		p_H 7·6.			p_H 7·6.			
	1 day.	2 days.	1 day.	2 days.	1 day.	2 days.	3 days.	1 day.	2 days.	3 days.	4 days.
Achromobacter 110	+	+++	++	+++	±	+	++	−	±	±	+
,, 131	++	+++	++	+++	±	+	+++	−	±	+	+
,, 180	++	+++	++	+++	±	+	++	−	±	+	+
,, 50	+	+++	+	+++	±	+	++	−	−	−	−
,, 100	+	+++	++	+++	±	++	+++	−	±	+	+
,, 147	+++	+++	+++	+++	+	++	+++	−	−	±	+
Pseudomonas 19	+++	+++	+++	+++	+	++	+++	−	+	+	++
,, 26	++	+++	++	+++	±	+	++	−	±	±	+
Bacillus 345	++	+++	+	++	−	−	−	−	−	−	−
,, 389	++	++++	++	++++	+	++	++	−	+	++	++

inhibition of growth. This is the explanation of the finding previously published (Coyne, 1933) that at 0° C. fresh fish can be kept in 50 per cent. carbon dioxide for at least twice as long as in air, a discovery which may be of economic importance. The increased efficiency of carbon dioxide at low temperatures is to be expected since the resistance of micro-organisms to growth inhibitory factors is naturally greatest at the optimum temperature.

That carbon dioxide exercises an even more effective inhibition on the growth of moulds has been shown by Tomkins (1932). Since the deterioration of flesh foods is in the main caused by the activities of bacteria and moulds it follows that storage of these foods in carbon dioxide gas should be of value. Favourable results have been reported by Killeffer (1930) on a variety of foodstuffs, by Moran, Smith and Tomkins (1932) on meat, by Callow (1932) on pork and bacon and by Coyne (1933) on fresh fish.

The mode of action of carbon dioxide is of interest. Fränkel (1889) observed that the effect could not be correlated with lack of oxygen since both facultative and strict anaerobes were affected. This has been abundantly confirmed. That the alteration in p_H of the medium caused by carbon dioxide is sufficient to inhibit bacterial growth has been maintained by several investigators, although Larson, Hartzell, and Diehl (1918) pointed out that this explanation could not account for the high death-rate of bacteria in carbonated waters.

Valley and Rettger (1927) produced evidence that those organisms which are most susceptible to acid are most affected by carbon dioxide and that in heavily buffered media inhibition of growth is less marked. They concluded "that any harmful action brought about on bacteria by the application of carbon dioxide is exerted through the increased H-ion concentration of the medium exposed to the gas." That the experimental data did not justify such a conclusion was remarked recently by Callow (1932) who noted that a number of the cultures showed less luxuriant growth in carbon dioxide than they did in air on a medium of the same hydrogen ion concentration. At the same time Coyne (1932) produced evidence that alteration in p_H of the medium did not suffice to explain the inhibition. In many cases growth in carbon dioxide is considerably poorer than in air on media of the same hydrogen ion concentration. The present investigations have shown also that the inhibition exerted by carbon dioxide on the growth of some bacteria is at least of the same degree in unbuffered (normal) and in highly buffered media in spite of wide difference in hydrogen ion concentration.

Carbon dioxide is well known to have a high penetrating power with regard to living cells of animals, plants, and protozoa. Jacobs (1920) tested on tad-

poles, protozoa, and taste receptors of the tongue solutions of various acids at the same p_H, and claimed that carbon dioxide had a higher penetrating power than any other acid and even than the hydrogen ion. Although the physiological action produced was caused by the hydrogen ion the reaction occurred internally and was specific in its nature. Jacques and Osterhout (1929) measured the rate of penetration of carbon dioxide into living cells of *Valonia* and found this to be independent of the external p_H (6·8 and 4·8). They concluded that little penetration of ions occurs and that the controlling factor is the external concentration of undissociated carbon dioxide no matter what variations occur in ionic concentration. It is possible that carbon dioxide acts directly on bacterial cells. If these cells are highly permeable towards carbon dioxide the change in intracellular p_H may be much greater than would be caused by growing organisms in an acid medium. There is evidence in support of this view.

Shaughnessy and Winslow (1927) studying the reactions of suspensions of *Bacterium coli* and of *Bacillus cereus* in distilled water and in presence of electrolytes, observed that the cells tended to maintain a favourable p_H by liberation of acid or alkali—mainly carbon dioxide and ammonia. Although the diffusion of these substances was affected by electrolytes the cells appeared to be relatively impermeable to ions, "the primary adaptive regulation being due to a balanced production of CO_2 and NH_3."

Such a mechanism would certainly break down under the influence of an atmosphere of carbon dioxide. It is unfortunate that the high solubility of ammonia renders impossible a comparison of the effect of an atmosphere of this gas. In this connection, however, the observations of Tomkins (1932, *a*) on the action of carbon dioxide on fungi are of interest. Tomkins differentiated the effect from that produced by such substances as chloroform, acetone, and ether vapour on the one hand, and acetaldehyde, hydrogen cyanide, and hydrogen sulphide on the other. He showed that carbon dioxide resembled most closely ammonia which in a previous communication (1932, *b*) he had placed in a unique position.

The delicately balanced physiological equilibrium of the bacterial cell may be disturbed by carbon dioxide independently of an effect on p_H. It is well known that *Brucella abortus* and *Lactobacillus acidophilus* grow better in presence of 5 per cent. to 10 per cent. carbon dioxide. The carbon dioxide requirements of many other bacteria have also been studied. After a comprehensive investigation Valley and Rettger (1927) concluded that "the removal of carbon dioxide from an environment otherwise favourable to

development results in complete cessation of bacterial growth." The ease with which inhibition could be induced varied considerably with different organisms.

These observations indicate that carbon dioxide has a definite function in cell metabolism and that each bacterial species has an optimal carbon dioxide requirement above and below which conditions are less favourable for growth. That those organisms whose normal habitat is the intestine or respiratory tract of animals are more resistant to high concentrations of carbon dioxide than saprophytic types would therefore be expected, and in general this is so. A study of the results of Birkinshaw, Charles and Clutterbuck (1931) on bacterial metabolism fails, however, to show any correlation between the amount of carbon dioxide produced from a synthetic medium by a large number of bacteria and the resistance of these species.

Variations in the permeability of the cells of different species of bacteria should be a factor in determining resistance either to high concentrations of carbon dioxide or to complete removal of this gas from the environment. Shaughnessy and Winslow state that the permeability of *Bacillus cereus* is probably much greater than that of *Bacterium coli* since the former dies rapidly in aqueous suspension while the latter is practically unaffected. In concordance with this observation *Bacterium coli* is strongly resistant to high concentrations of carbon dioxide while *Bacillus cereus* is amongst the more susceptible organisms. On the other hand Valley and Rettger place their strain of *Bacillus cereus* in the same group as the majority of strains of *Bacterium coli*, as regards resistance to complete removal of carbon dioxide from the atmosphere. A lower optimum concentration of carbon dioxide would therefore appear to be desirable for *Bacillus cereus*.

It has been suggested previously by Callow (1932) and by Coyne (1932) that carbon dioxide may have a specific effect on some enzyme system in the bacterial cell. To test this hypothesis Haines (1933) has undertaken a study of bacterial dehydrogenases in presence of carbon dioxide, using the methylene blue technique. Conclusive results have not yet been obtained.

It would appear that a variety of factors affects the influence of carbon dioxide on bacteria. Alteration in the hydrogen ion concentration of the medium is not of primary importance, since the permeability of cells to carbon dioxide renders possible a change in intracellular p_H not dependent on the external p_H. Variations in the cell permeability of different genera, species, strains, and individual organisms will produce a graded effect. This explains partially the difficulty found in duplicating results and the fact that in many

cases where carbon dioxide does not produce complete inhibition of growth only a few isolated colonies develop. Inside the cell the effect of carbon dioxide may be due to an alteration in p_H, to an independent interference with any of the enzyme systems, or to a combination of these. Carbon dioxide is not merely an end-product of metabolism but has a definite physiological function in the bacterial cell.

This investigation has been carried out under the joint auspices of the Department of Scientific and Industrial Research (Food Investigation Board) and Imperial Chemical Industries Ltd., to whom the author is indebted for permission to publish the results.

Summary.

(1) The growth of a number of bacteria in the presence of different amounts of carbon dioxide (5 per cent. to 100 per cent.) at 0°, 10°, 25°, and 37° C., has been investigated.

(2) In general carbon dioxide exercises an inhibitory action on growth, varying from complete inhibition in some cases to an almost negligible effect in others. At temperatures below the optimum for growth the influence of carbon dioxide is invariably more marked.

(3) In 20 per cent. carbon dioxide at 0° C. growth of those bacteria responsible for the spoilage of fresh fish is almost completely inhibited.

(4) Alteration by carbon dioxide of the p_H of the culture medium does not suffice to explain these results. A marked intracellular change in p_H may result from the high permeability of living cells to carbon dioxide, and interference with cell enzyme systems may also be a contributory factor.

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Cytological Changes in the Lachrymal Gland Following the Administration of Certain Drugs.

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[Plates 8–9.]

During the course of an experimental investigation into the secretory mechanism of the lachrymal gland we were impressed by the varying responses of the gland to different drugs. Some of these substances produced an activity which lasted for a considerable period while others gave rise to a very transitory secretion. In addition we noted the fact that the response of the gland to pilocrapine injections was by no means identical with that produced by stimulation of the lachrymal nerves. We were compelled therefore to study the locus of action of each particular drug which had been used.

The presence of "secretory" granules in the lachrymal gland has been recorded by many observers (see Sundwall, 1926, for review of literature), but apparently little critical work has been carried out on the actual relation of these cytoplasmic inclusions to lachrymal secretion. In his monograph on "The Mitochondrial Constituents of Protoplasm," Cowdry (1918) discussed all the methods which had been used for the study of mitochondria and cytoplasmic granules. He came to the conclusion that the fuchsin-methyl green method as modified by Bensley (1911) presented the fewest objections, and in consequence we adopted this method for our fixed material. We were

influenced in this decision by Goetsch's work (1916) on the thyroid gland in which he was able to find a specific relationship between glandular activity and the mitochondrial content.

By the use of the above method we hoped to be able to determine the exact locus of action of the drugs we had used and to evaluate their relative effects. The method, we found, was by no means simple and required considerable practice before constant results were obtained. Hardening of the tissues gave rise to much difficulty in obtaining flat sections and the method of differentiation was found to be exceedingly difficult. Yet another obstacle was encountered. On otherwise perfect sections, red-stained "processes" were observed on the surface of the gland alveoli. Later we determined that these "processes" were portions of the cytoplasm of myo-epithelial cells surrounding each alveolus. The sections used were five microns in thickness and were observed by white transmitted light and by light passed through a green filter (Wratten No. 58 B.B.). In this way photography and identification of the red-stained material within the cells was rendered relatively simple.

In another series of experiments, one of us (B.) has studied the living cells of the gland, in order to compare the refractile bodies seen in them with the stained objects in the fixed material. These results will be reported later.

The Structure of the Normal Gland.

An examination of normal gland tissue stained in the above manner demonstrated the presence of small red-staining granules scattered throughout the cytoplasm of the alveolar cells. They were uniform in size and were not concentrated about the nucleus, fig. 1, Plate 8. A study of the cells stained supravitally with Janus green B confirmed the impression that they were mitochondria. The alveolar cavities were found to enter ducts, fig. 2, Plate 8, lined by a columnar epithelium. The cytoplasm of the epithelial cells of these ducts contained red-staining bodies varying in size from those found in the alveolar cytoplasm to masses almost as large as the nucleus. In fig. 2 these black rounded bodies are seen to have a close relationship to the nuclei which are stained green and are recognized easily by the nucleolar dot. Smaller particles are found in the cytoplasm towards the lumen. There was thus sufficient cytological difference between the two glandular epithelia to enable them to be readily recognized. The exact nature of the various particles in the duct cells has yet to be determined.

Experimental Technique.

In order to eliminate any possibility of irritation of the conjunctiva and the reflex lachrymation, di-allyl-barbituric acid and urethane was used as the anæsthetic. This drug injected into the peritoneal cavity produced complete anæsthesia within 15 minutes. Immediately muscular relaxation was well marked the femoral vein was exposed and a known quantity of drug injected. Five minutes later the glands were excised and fixed in Regaud's mixture, or fixed *in situ* by the injection of Regaud's mixture into the carotid artery and removed later. No appreciable difference was noted in the histological appearances of the glands fixed by these two methods. In four animals the glands were not removed until after varying periods (30 minutes, 1 hour, 1 hour 30 minutes, and 2 hours) from the time of injection of the drug. This was done to obtain evidence of recovery.

Material.

Cats were used throughout this series of experiments.

Drug.	Dose.	No. of experiments.
Control animals	—	2
Acetyl choline	0·5 mg.	2
Ergotamine	1·0 mg.	2
Histamine	0·5 mg.	2
Physostigmine	0·25 mg.	2
Physostigmine	0·5 mg.	6
Acetyl choline	0·5 mg.	
Pilocarpine	1·0 mg.	2
Pituitrin (surgical)	0·25 c.c.	2

In another series dogs were used, but as there is considerable difference in gland histology between the two species, consideration of the effects in these animals will form the subject of another paper.

Observations.

Acetyl Choline.—The alveoli, fig. 3, Plate 8, showed signs of fine vacuoles scattered throughout the cytoplasm. Mitochondria were still present. In the ducts, fig. 4, Plate 8, the large globular bodies disappeared and in their place small granules were observed. The dark spherical bodies in figs. 3 and

4 are nuclei and not globules of red-staining material. Note the darkly stained myo-epithelial processes on the left of the alveolus in fig. 3.

Ergotamine.—In a previous experiment we had observed that ergotamine caused a copious lachrymation. The exact mechanism of this effect is unknown. The alveoli showed no appreciable cytological change, fig. 5, Plate 8. The duct, fig. 6, Plate 8, demonstrated the loss of globular bodies and the presence of a coagulable material within the lumen.

Histamine.—Figs. 7 and 8, Plate 8, there was found no sign of activity within the cytoplasm of either the alveoli or ducts. There was, however, marked evidence of narrowing of the lumen in each alveolus. This was caused not by an increase in the radial diameter of the gland cells but probably by the contraction of the myo-epithelial cells.

Physostigmine.—The marked vacuolation of the alveoli, fig. 9, Plate 9, was very striking. The mitochondria were present. In fig. 10, Plate 9, the duct shows marked flattening of the epithelial cells. The larger granules were found near the smaller ones near the nucleus. The globular bodies had disappeared or had broken up into large granules.

Physostigmine and Acetyl Choline.—The fine foamy appearance of the alveoli, fig. 11, Plate 9, is readily observed. The nuclei were packed closely against the basement membrane and the cell walls distinctly outlined. The mitochondria were seen to lie on the surfaces of the vacuoles but are impossible to photograph owing to the vacuolation. The ducts showed an almost complete loss of granular substance; only a few large granules remained in them, fig. 12, Plate 9. The nuclear outlines were quite visible.

Pilocarpine.—The effect of pilocarpine on the alveoli was to form a coarse foam in the interstices of which the mitochondria were observed in approximately normal numbers, fig. 13, Plate 9. The epithelium of the gland ducts was flattened but large globular bodies and coarse granular material were still present, fig. 14, Plate 9.

Pituitrin.—Administration of pituitrin caused no effect on the alveoli but a marked action on the ducts. These structures were compressed, the nuclei being deformed and the cell outlines rendered more conical. No effect on the granular material was observed, fig. 15, Plate 9.

Recovery Phase in a Gland Duct.

The granular material was observed as large dark staining granules closely applied to the nuclear membrane. In some ducts no other granular matter

was observed. Fig. 16, Plate 9, demonstrates the presence of the peri-nuclear granular masses and also less well stained and smaller granules near the lumen, two hours after a dose of 0·5 mg. of acetyl choline and 0·5 mg. of physostigmine. A study of the recovering gland shows that at the end of one hour only peri-nuclear granular material is present. Later phases may show only such granules or in addition to them granular material distributed towards the periphery. At the end of two hours no large globular bodies had appeared although there was a marked increase in the number of the coarse granules packed closely together near the nuclear membrane. It is possible that the globules are formed by the fusion of these large granules.

Discussion.

Sundwall (1916) has summarized the literature on the cytoplasmic structure of the lachrymal gland and has added to this a mass of original observations, mainly on the lachrymal gland of the ox. Sundwall confirmed, in the ox, the presence of highly refractile " granules " in the alveolar and duct epithelium.

We noted that the mitochondria in the alveoli and the " granular " material in the duct cells are not preserved in alcohol, Bouin's or Zenker's fluid or in a formol-mercuric chloride fixative. If Bensley's acetic-osmic-bichromate mixture (1911) or Regaud's formalin-bichromate fixative is used the granules are well preserved and can be stained by Altmann's aniline-fuchsin and differentiated with methyl green.

It is obvious that the alveolar granules must be regarded as mitochondria and it is hard to believe that such structures are not present within the duct cells. They may be the smaller granules which can be observed in most of the figures. However, it is difficult to understand at present why, in the alveoli the mitochondria should persist, while in the duct cells they disappear, when the gland is activated by acetyl choline and physostigmine. The larger bodies—globules and coarsely granular material—seem to be associated closely with the nuclear membrane and it is probable that they are entirely different morphologically. Their absence is related undoubtedly to glandular activity. These bodies seem, in our opinion, to be the only structures which justify the name of " secretory granules."

Reviewing our observations, it is apparent that one must consider the effects on the alveoli apart from those produced on the duct cells. The alveoli show as a result of activation a vacuolation of the protoplasm which may be " fine " or "coarse." Pilocarpine causes a coarse vacuolation, but produces no change

in the mitochondrial content. Acetyl choline and physostigmine acting alone or together produce a finer vacuolation with no observed mitochondrial change. When the lachrymal nerves are stimulated and the gland made to secrete, exactly similar effects are observed in the alveoli.

A study of the duct cells demonstrates that activity of these cells is caused by acetyl choline and physostigmine. Pilocarpine has no effect on the granules or globules of the duct cells. The maximal effect is produced by a combination of the two drugs—acetyl choline and physostigmine. Full activity as judged by the almost complete loss of granules is not accompanied by vacuolation of the duct cells.

During the course of our experimental work we noted that ergotamine in doses of 1/3 milligram per kilo. of body weight caused in some animals a profuse lachrymation provided that the animal was given the drug immediately after anæsthesia had been produced by a barbituric acid anæsthetic. If the administration of the ergotamine was delayed for half an hour or more no effect was obtained. We have not investigated this point, but the experiments of Keeser and Keeser (1927) suggest a possible explanation. The barbiturates according to these observers probably act on thalamic and hypothalamic nuclei. It is possible then that ergotamine may stimulate cells of the hypothalamus or thalamus causing lachrymation. The abolition of the lachrymatory phenomena when the anæsthesia is prolonged for more than about half an hour may be due to a " block " of these cells.

The lachrymation produced by ergotamine is apparently an effect mainly on the ducts as there is no apparent change in the alveoli. Moreover, the duct cells show loss of granular matter and almost invariably a coagulum which stains faintly pink, is found in the duct lumen. This is of interest as we have not observed any coagulum in the ducts in those experiments in which pilocarpine, acetyl choline and physostigmine were used.

Histamine and pituitrin give rise to lachrymation of an entirely different character to that already considered. Histamine causes the expulsion of a few drops of tears within a few seconds of administration. When the gland is examined there are no signs of glandular activity. The lumina of the alveoli are markedly narrowed and may be obliterated. We attribute this effect to the contraction of the myo-epithelial cells of the alveoli. Pituitrin gives rise to a very different picture. The alveoli are quite normal in their appearance but the ducts show evidence of marked contraction in diameter. The nuclei are deformed into ellipsoidal bodies and are no longer spherical. This appearance is noted only in those ducts which possess a muscular coat.

The regeneration of the cytoplasmic granules in the ducts apparently takes place close to the nuclear membrane. The small bodies apparently run together and may eventually form globular masses. Regeneration in the alveoli consists of a gradual reduction of the size of the vacuoles and as these disappear the mitochondria are seen to lie free in the cytoplasm. In no case have we observed alveolar cells which did not contain mitochondria.

Our results indicate that the true secretory activity of the lachrymal gland is the result of stimulation of either the alveoli or the ducts or of both structural units. The alveoli seem to be responsible for the secretion of large quantities of watery fluid (*e.g.*, with pilocarpine) while the ducts secrete a fluid which probably contains a higher proportion of solids (*e.g.*, with ergotamine).

The effect of acetyl choline and the synergic effect of physostigmine closely parallel the observed effects caused by nerve stimulation (lachyrymal nerves). One must conclude therefore that, in view of the experimental work of Dale and Gaddum (1930), Babkin (1932) and Baxter (1932), the parasympathetic innervation of the gland produces glandular activity by the release of specific substances similar to acetyl choline at the nerve-endings. Pilocarpine is therefore not a drug which reproduces effects, identical with parasympathetic stimulation, in the lachrymal gland. It is interesting to compare these observations with those of Baxter (1932) on the variations of the solid content of saliva produced by pilocarpine and choline. With the former drug a very "watery" saliva was obtained while with the latter the percentage of the organic and mineral content showed a marked increase.

The secretion, obtained by the use of histamine, is probably due to the activity of myo-epithelial tissue and not to any real glandular secretion. The effect is so transient and the secretion so scanty that it can be compared with that obtained by Babkin and Mackay (1930) and Mackay (1929) in the salivary glands. We found no evidence of a Diplosoma or contractile mechanism within the alveolar cells such as Zimmermann (1927) believes to be present within secretory cells of the salivary glands. In lachrymal glands stained by other methods we found no smooth muscle cells around the alveoli such as are present in the walls of the secretory ducts. On the other hand we had ample evidence of the "Korbzellen" of Zimmermann (1927). These are the myo-epithelial elements which lie within the basement membrane of the alveoli.

The small amount of secretion produced by pituitrin is due to the contraction of the smooth muscle of the ducts alone as in such experiments no change in the shape of the alveolar lumen was noted. Distortion of the lumen of the ducts was only observed in the pituitrin experiments.

Whitnall (1932) states that "by the contraction of the protoplasmic network of the cell, the secretion is expressed into the central canal of the acinus and thence passes into the excretory canals." We have no evidence that such a contraction takes place within the alveolar cells. Rather does it seem to us that pilocarpine and acetyl choline cause an increased permeability of the alveolar cell walls to fluid which is passed rapidly through the cell and secreted at the lumen. We can offer no explanation at present of the behaviour of the duct cells.

Conclusions.

(1) There are two separate secretory units within the lachrymal gland of the cat—the alveoli and the secretory ducts.

(2) The alveoli react to pilocarpine, acetyl choline and physostigmine by undergoing extensive vacuolation without loss of mitochondria.

(3) The secretory ducts never show vacuolation by any method of stimulation. Their granular material disappears during stimulation and is reconstituted again mainly around the nuclear membrane.

(4) The secretions caused by histamine and pituitrin are due to the activity of contractile elements around the alveoli and secretory ducts.

(5) The drugs which reproduce the effects of nerve stimulation are acetyl choline and physostigmine and are therefore truly parasympathetico-mimetic for the lachrymal gland.

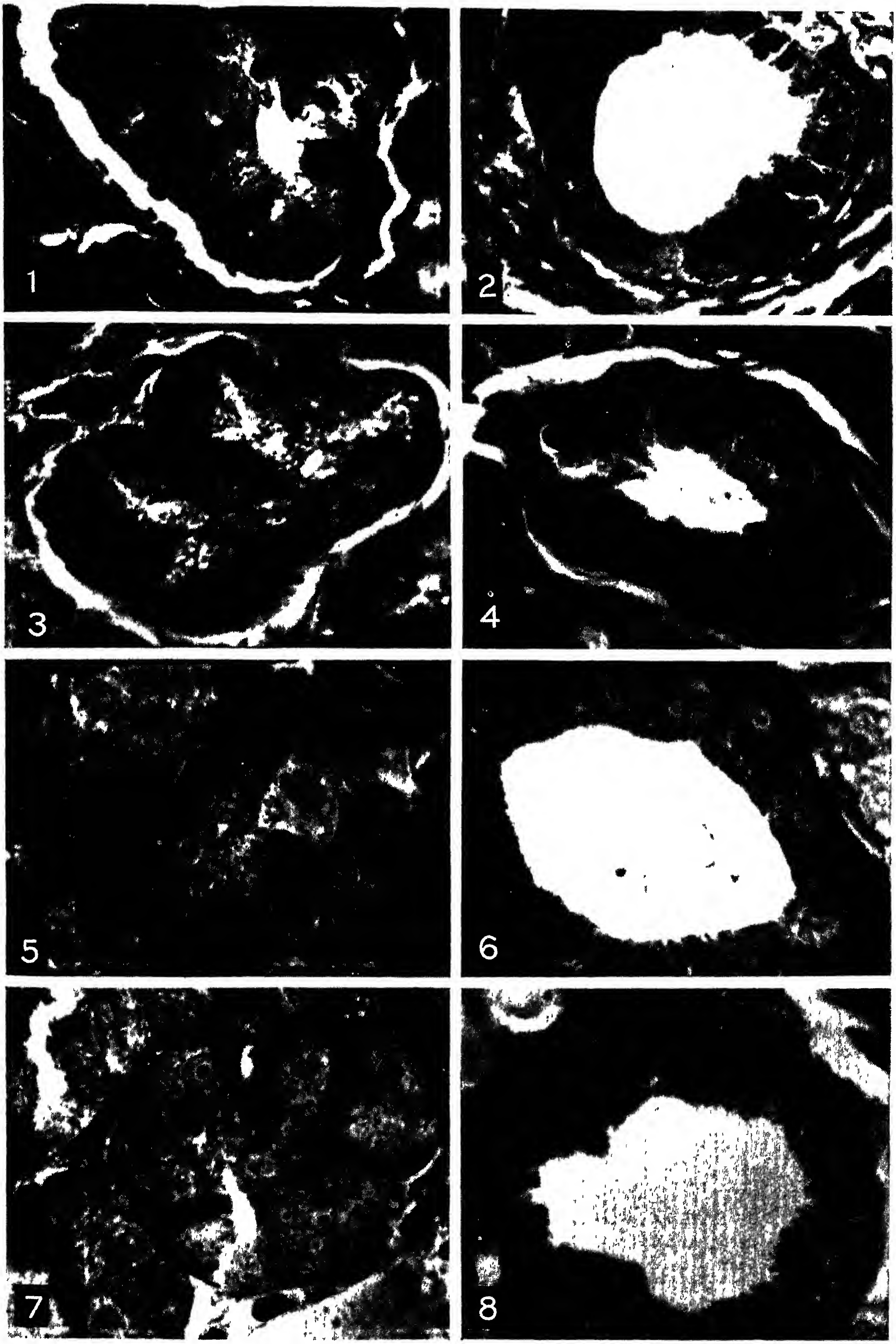
We wish to extend our thanks to the Banting Research Foundation for the grant made to one of us (McD.) to aid in this work. To Professor F. E. Lloyd of this University we offer our thanks for his generous assistance in photographing our material.

DESCRIPTION OF PLATES.

The figures reproduced in the plates are untouched photographs of sections of cats' lachrymal glands. The sections were cut five microns in thickness and stained by Cowdry's modification of Altmann's anilin-fuchsin-methyl green stain. Photographs were made with the following lens combinations :—Ocular (Zeiss) × 8, objective 120 (Zeiss). The magnification in the plates is approximately 1100 diameters.

PLATE 8.

FIG. 1.—Normal control gland alveolus to show the distribution of the red-staining granules—mitochondria—in the cytoplasm. The animal was anaesthetized by di-allyl-barbituric acid.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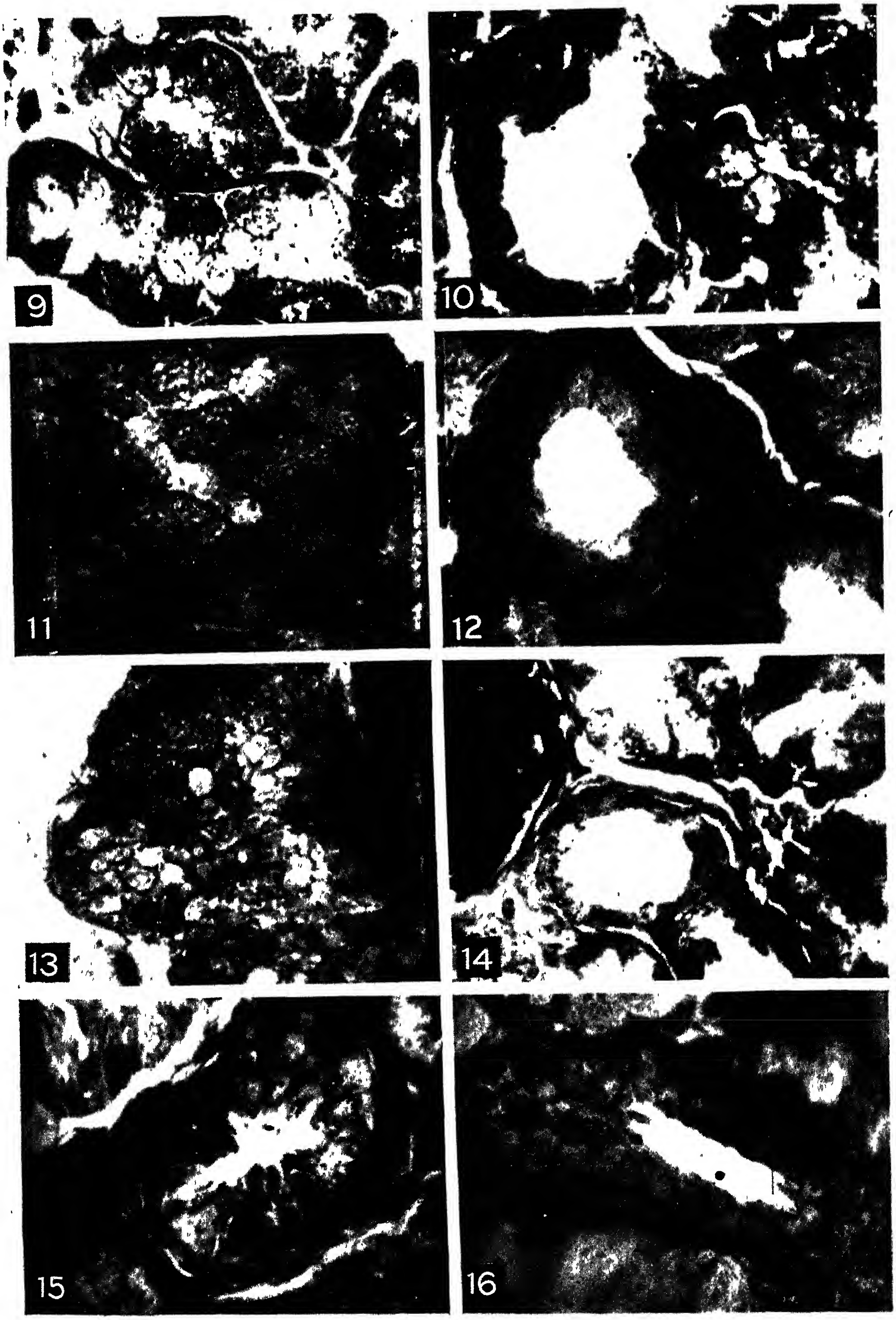
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FIG. 2.—Normal control intralobular duct illustrating the varying sizes of the red-staining cytoplasmic granules and globules. The nuclei were stained green and show faint nucleoli. From the same section as fig. 1.

FIG. 3.—Alveolus. Five minutes after the injection of 0·5 mg. acetyl choline. Note the finely vacuolated cytoplasm and the dark mitochondria. The nuclei are the dark spherical bodies near the basement membrane.

FIG. 4.—Secretory duct from the same section as fig. 3. Note the absence of large globular bodies and the reduction in the number of coarse granules. The dark spherical bodies are nuclei.

FIG. 5.—Alveolus. Five minutes after the injection of 1·0 mg. ergotamine tartrate. Normal mitochondrial distribution and absence of vacuoles.

FIG. 6.—Secretory duct from the same gland as fig. 5. Note the coagulum in the duct lumen, the reduction in number of the large granules and absence of globules.

FIG. 7.—Alveolus. The effect of 0·5 mg. of histamine. The gland was removed 5 minutes after injection of the drug. Normal distribution of mitochondria. No vacuoles present.

FIG. 8.—Secretory duct from the same gland as in fig. 7. Note the large globules and granules of varying sizes.

PLATE 9.

FIG. 9.—Alveolus. Five minutes after injection of 0·25 mg. of physostigmine. Note the vacuolation of the cytoplasm and the presence of mitochondria.

FIG. 10.—Secretory duct from same animal as in fig. 9. The duct epithelium is flattened; the globules are absent but there is a reduction of the granular content.

FIG. 11.—Alveolus. Seven minutes after the injection of 0·5 mg. of physostigmine and 0·5 mg. of acetyl choline. Note the vacuolation of the cytoplasm. The mitochondria are present.

FIG. 12.—Secretory duct from the same gland illustrated in fig. 11. Note the absence of vacuolation and an almost complete loss of granules. A few larger granules are present.

FIG. 13.—Alveolus 5 minutes after the injection of 1·0 mg. of pilocarpine. Note the large vacuoles and well-stained mitochondria.

FIG. 14.—A small secretory duct from the same gland as fig. 13. The duct cells are flattened, but there is no apparent discharge of granules.

FIG. 15.—Five minutes after the injection of 0·25 c.c. of "surgical" pituitrin. The contractions of the smooth muscle has compressed the cells distorting the nuclei and altering the shape of the globules. Note the spindle-shaped "globule."

FIG. 16.—Secretory duct, 2 hours after the injection of 0·5 mg. of acetyl choline and 0·5 mg. of physostigmine. Compare fig. 12. Granular material is being reconstituted around the nuclear membrane especially near the basal pole of the nucleus.

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The Action of Radium on Cancer Cells. I.—*Effects of Hydrocyanic Acid, Iodo-acetic Acid, and Sodium Fluoride on the Metabolism and Transplantability of Cancer Cells.*

By H. G. Crabtree and W. Cramer.

(Communicated by J. A. Murray, F.R.S.—Received May 5, 1933.)

In previous investigations (Cramer, 1932; Crabtree, 1932) evidence was obtained that—

(*a*) susceptibility to radium is not a fixed property of a given type of cancer cell, but varies with changes in the environment. The success of transplanatation of sections of mouse tumour, irradiated *in vitro*, depended on the nature of the saline medium in which they were suspended;

(*b*) a primary effect of radium radiation on cells *in vitro* was a selective diminution in their respiration, while glycolysis remained comparatively constant.

These observations opened up a new line of investigation on the action of radium on cancer cells. A systematic study has therefore been undertaken of the effects which changes in the environment have on the susceptibility of malignant cells to radiation. In the second paper of this series, experiments are recorded which show the varying sensitivity to radium of tumour cells treated with well-known inhibitors of metabolism.

Slices of tumour tissue, suspended in saline media modified by the addition of hydrocyanic acid, iodo-acetic acid or sodium fluoride, were irradiated and

the effects produced were assessed by the subsequent growth or non-growth of the transplanted cells. The necessary non-irradiated control tissues were kept in similar media during the experimental period, and it was essential that these controls should suffer no permanent damage by the treatment. This implies that the effects of the inhibitors of metabolism should be completely reversible.

Scanty data were available on this point, particularly on the degree of reversibility found when varying concentrations of these inhibitors had acted on tumour cells for periods corresponding to the times used in the radiation experiments.

This preliminary investigation includes a study of :—

(*a*) the inhibitions of the respiration and glycolysis of tumour tissue produced by a wide range of concentrations of hydrocyanic acid, iodo-acetic acid and sodium fluoride ;

(*b*) the degree of reversibility of the action of these inhibitors ;

(*c*) the effect of these substances on the viability of the cell as measured by the behaviour of tumour cells transplanted after treatment.

Experimental Section.

1. *Hydrocyanic Acid.*—The degree of checking of respiration by small concentrations of hydrocyanic acid has been a subject of controversy for many years. Warburg (1931) restates his earlier finding that 100 per cent. checking is obtained in CO_2-bicarbonate buffers, and recent evidence is brought forward by Alt (1930) who found, with rat kidney, liver, and spleen, an almost complete inhibition of respiration with M/100 HCN. A concentration of M/500 checked the respiration to the extent of 80 per cent. to 90 per cent.

These results are in contrast to the smaller checkings found by Dixon and Elliott (1929) for the same tissues suspended in phosphate buffers.

The method used in this work was that introduced by Warburg (1926), in which two slices of tissue are placed in two vessels containing different amounts of a Ringer-glucose-bicarbonate medium with 5 per cent. CO_2 and 95 per cent. O_2 as the gas phase. The accuracy of this method rests on the assumption that the tissue-sections used are uniform in the two vessels. With normal tissues, using adjacent sections, this possible source of error can be ignored, since large numbers of experiments give perfectly uniform results. With tumour tissue, often specked with visible necrosis and showing local degeneration microscopically, the errors introduced may be large. For this

reason, in all the work detailed here, measurements were made in duplicate or triplicate and only those accepted which showed a close correspondence in the figures obtained.

Two experimental methods are possible :—

(*a*) The method described later in studying the effects of iodo-acetic acid and fluoride, where vessels with side-bulbs containing the cyanide are used. After a preliminary determination of the initial metabolism, the cyanide is tipped into the main vessel, and thus the same tissues are used as controls. This method failed to give any sort of consistency in the results. If untreated KCN were used, the alkali present disturbed the experiment so as to give meaningless results. With cyanide neutralized with HCl, there was always less apparent checking than was found by method (*b*). It seemed possible that HCN vapour was passing from the side-bulb to the main vessel during the preliminary measurement of normal metabolism.

(*b*) The tissues are placed immediately into Ringer-glucose-bicarbonate containing the required concentration of HCN, made by adding a solution of KCN brought to $p_H = 7 \cdot 4$ by addition of HCl. Separate control tissues are required in this experiment, and the possibility of error is increased. Yet this method was used and gave consistent figures.

It is impossible to state with certainty that there is 100 per cent. checking in any case. The effect of hydrocyanic acid on the final 10 per cent. of respiration proves indeterminate, since it is within the limits of error of the technique. Occasionally calculation shows 100 per cent. checking, but the majority of experiments show 85 to 95 per cent. This checking is attained by M/1000 HCN, and further increase in the concentration has led to substantially the same result.

During the period when the respiration of tumour tissue is largely checked by HCN, aerobic glycolysis rises to a figure approaching that of anaerobic glycolysis, as first pointed out by Warburg (1926).

Whatever the concentration of HCN used, M/1000 to M/20, no obvious damage is inflicted on the tumour tissue over a period of 1 hour.

On removal of the tissue to the ordinary Ringer-glucose-bicarbonate solution its metabolism quickly returns to normal. The complete reversibility of hydrocyanic acid checking is also shown by the transplantation of tissues kept for 1 hour in concentrations as high as M/20.

Since the metabolism results are so similar for all concentrations of HCN, only one experiment is represented in fig. 1. The corresponding transplantation experiments are shown in fig. 2.

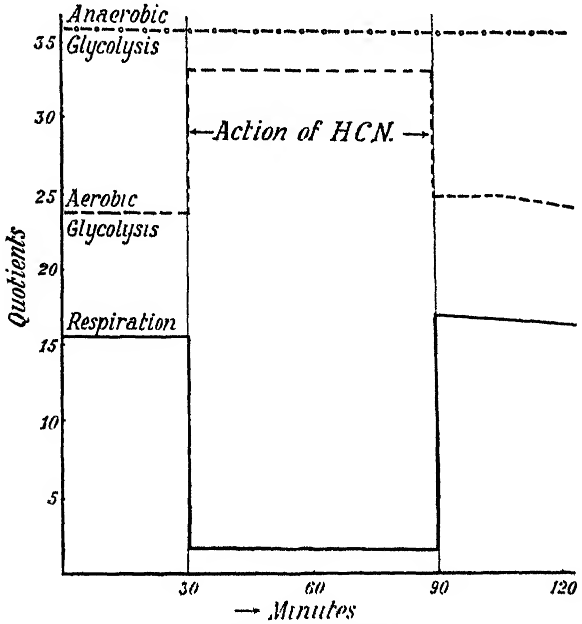


FIG. 1.—Effect of M/500 HCN on the respiration and glycolysis of tumour tissue, showing subsequent recovery.

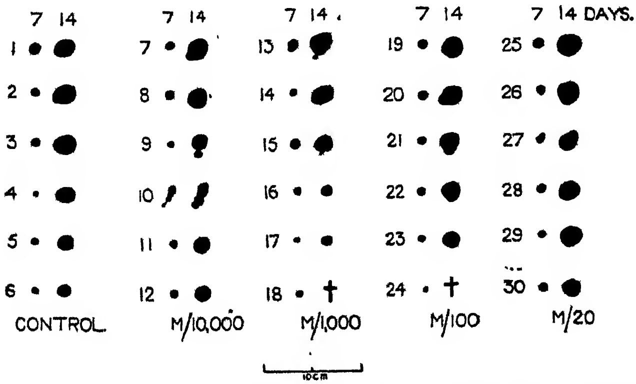


FIG. 2.—HCN in varying concentrations for 1 hour. HCN has no damaging effect on cancer cells in concentrations up to M/20.

2. *Iodo-acetic Acid.*—Little work on the effect of this acid on the metabolism of tumour tissue is available, and this deals mainly with the early inhibitions produced and not with the later recovery. Harrison and Mellanby (1931)

confined their studies to the inhibition produced in the glycolysis of tumours. Krebs (1931) worked with Jensen rat sarcoma, rat brain and testis. He found in all these tissues a similar behaviour in the presence of small concentrations of iodo-acetic acid, the well-known checking of glycolysis being accompanied by a parallel checking of respiration of the same order. Addition of lactate or pyruvate restored the respiration temporarily to its normal value. Meyerhof and Boyland (1931) and Needham (1932) found a similar restoration of respiration by lactate for amphibian muscle and chick embryos respectively.

This suggests that the checking of respiration is a secondary effect, owing to the diminution of products of intermediate metabolism which are suitable substrates for oxidation.

Two types of experiment were carried out, using the Ringer-glucose-bicarbonate medium throughout:—

(1) Measurement of initial metabolism in two vessels with side-bulbs containing an amount of neutralized iodo-acetic acid which gave the required concentration on tipping into the main vessel. After about 30 minutes, the acid was added and readings on the manometer recommenced after a short interval.

(2) For the study of the recovery metabolism, two slices of tumour tissue were kept shaking in a known concentration of iodo-acetic acid in Ringer-glucose-bicarbonate for periods of ½ to 1 hour. These were then removed, washed repeatedly, and their metabolism measured. A separate control was used to give the initial metabolism. Tissues treated in this manner were also transplanted into mice to test their viability.

Typical results for various concentrations are given in Tables I and II, and fig. 3 shows the result of transplantation experiments. In figs. 4 and 5 the combined results of inhibition and recovery experiments are demonstrated, the respiration and glycolysis effects being shown separately for the sake of clearness.*

* The original symbols used by Warburg (1926) have been used in all the tables, and are defined as follows:—

$$Q_{O_2} = \text{respiration} = \frac{\text{c.mm. } O_2 \text{ absorbed}}{\text{mg. tissue (dry)} \times \text{hours}}.$$

$$Q_M^{O_2} = \text{aerobic glycolysis} = \frac{\text{c.mm. } CO_2 \text{ evolved by lactic acid formation in } O_2}{\text{mg. tissue (dry)} \times \text{hours}}.$$

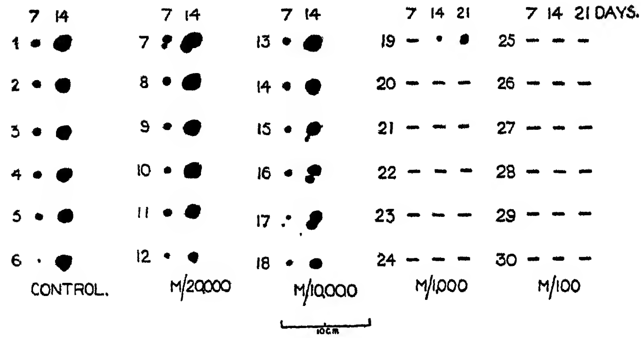
$$Q_M^{N_2} = \text{anaerobic glycolysis} = \frac{\text{c.mm. } CO_2 \text{ evolved by lactic acid formation in } N_2}{\text{mg. tissue (dry)} \times \text{hours}}.$$

Table I.—Inhibiting Effects of Various Concentrations of Iodo-acetic Acid on Tumour Metabolism.

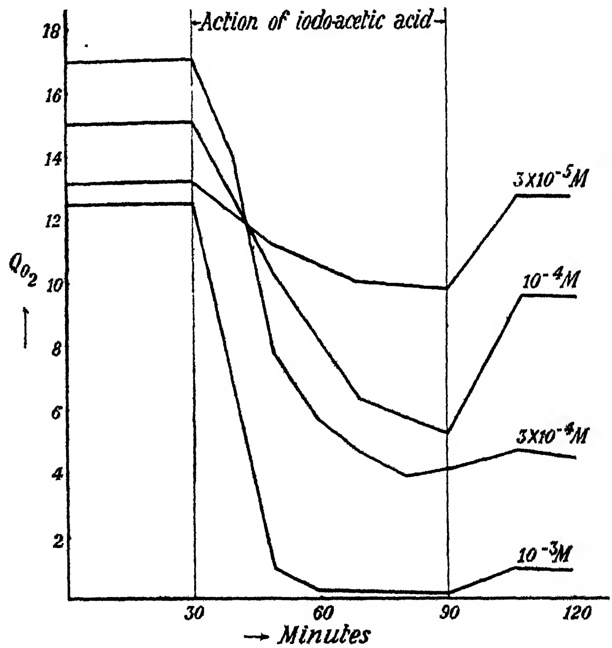
Tumour.	Concentration of iodo-acetic acid.	Initial metabolism.			Metabolism after addition of iodo-acetic acid.				Per cent. fall in Q_{O_2}.	Per cent. fall in $Q_M^{O_2}$.	Per cent. fall in $Q_M^{N_2}$.
		Q_{O_2}.	$Q_M^{O_2}$.	$Q_M^{N_2}$.	Time.	Q_{O_2}.	$Q_M^{O_2}$.	$Q_M^{N_2}$.			
					min.						
2146	3×10^{-5} M	17·7	19·4	32·6	30	12·7	10·3	22·8	28	47	30
					30	11·0	8·8	19·3	38	55	41
J. R. S.	3×10^{-5} M	13·0	22·9	35·6	20	11·2	17·9	25·3	14	22	29
					20	9·8	15·9	23·2	25	30	35
					20	9·6	14·9	21·8	26	35	39
2146	10^{-4} M	14·9	15·4	33·1	20	10·5	8·7	15·1	30	44	54
					20	6·3	3·8	10·1	58	75	69
					20	5·0	2·6	6·9	67	84	79
J. R. S.	10^{-4} M	17·7	17·1	—	20	8·5	4·9	—	52	71	—
					20	4·5	4·9	—	75	71	—
					20	4·0	3·6	—	78	80	—
2146	3×10^{-4} M	17·2	16·5	—	30	9·1	2·3	—	47	86	—
					30	2·3	1·1	—	87	93	—
J. R. S.	3×10^{-4} M	16·8	22·9	32·3	10	13·8	1·8	6·6	18	92	80
					10	7·8	1·5	5·5	54	93	83
					10	5·7	0	3·2	66	100	90
					10	4·6	0	2·6	73	100	92
					10	4·0	0	2·0	76	100	94
					10	4·2	0	2·3	75	100	93
J. R. S.	10^{-3}	12·2	23·2	32·8	10	1·9	0	0	84	100	100
					10	0·9	0	0	93	100	100
					10	0	0	0	100	100	100

Table II.—Experiments showing Recovery of Metabolism of Tumour Tissues kept in varying Concentrations of Iodo-acetic Acid.

Tumour.	Time of inhibition.	Concentration of iodo-acetic acid.	Initial metabolism.			Recovery metabolism.			Per cent. recovery of Q_{O_2}.	Per cent. recovery of $Q_M^{O_2}$.	Per cent. recovery of $Q_M^{N_2}$.
			Q_{O_2}.	$Q_M^{O_2}$.	$Q_M^{N_2}$.	Q_{O_2}.	$Q_M^{O_2}$.	$Q_M^{N_2}$.			
	min.										
2146	30	3×10^{-5} M	18·4	17·6	32·3	16·0	13·0	30·1	87	74	93
2146	60	3×10^{-5} M	17·7	19·4	—	16·8	16·4	—	95	85	—
J.R.S.	60	3×10^{-5} M	14·3	17·8	34·6	11·9	15·2	30·2	82	85	87
2146	30	10^{-4} M	17·1	18·8	34·1	11·6	7·7	22·0	68	41	65
J.R.S.	60	10^{-4} M	17·1	20·2	36·5	10·8	10·1	25·2	63	50	69
2146	30	3×10^{-4} M	17·2	16·5	—	4·1	1·1	—	24	7	—
2146	60	3×10^{-4} M	17·2	19·8	36·3	5·0	1·6	9·1	29	8	25
J.R.S.	60	3×10^{-4} M	12·6	21·3	33·5	2·9	1·3	7·3	23	6	22
J.R.S.	60	10^{-3} M	14·2	18·6	30·8	1·0	0·6	2·8	7	3	9



3.—Iodo-acetic acid in varying concentrations for 1 hour. Iodo-acetic acid has a lamaging effect on cancer cells which is very pronounced, in concentrations of M/1000 .nd slight but still noticeable in a concentration of M/10000. With a concentration of M/20000 there is complete recovery.



. 4.—Effect of iodo-acetic acid on the respiration of tumour tissue, showing inhibition and subsequent recovery.

The following points may be noted :—

(a) Except in the case of the higher dilutions of iodo-acetic acid, there is a progressive fall in respiration throughout the experimental period. Initially there is a large checking of glycolysis which is accompanied

by a much smaller fall of respiration. The respiration, however, progressively decreases until its value approaches that of the checked glycolysis. With a concentration of 10^{-3} M there is an almost instantaneous checking of both respiration and glycolysis.

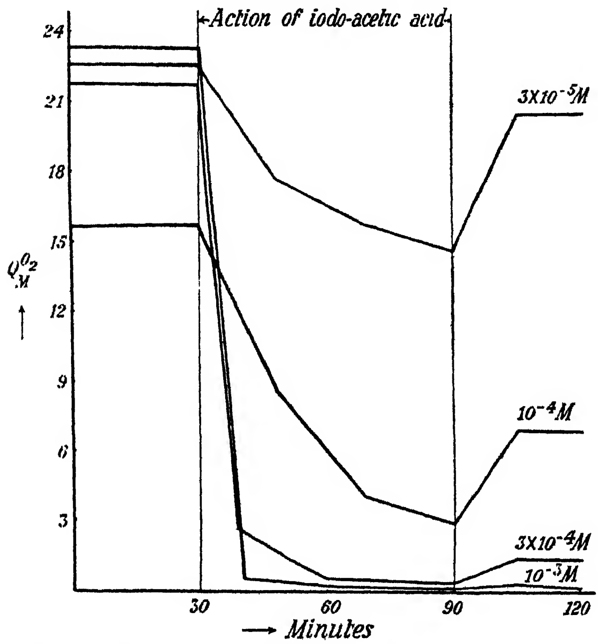


FIG. 5.—Effect of iodo-acetic acid on the aerobic glycolysis of tumour tissue, showing inhibition and subsequent recovery.

(*b*) With 3×10^{-5} M iodo-acetic acting over 1 hour, there is a complete metabolic recovery. With increasing concentrations this recovery becomes less and less, and with 10^{-3} M, the treatment has practically killed the cell. Thus the specific, reversible effect of iodo-acetic acid is limited to very small concentrations beyond which general toxic effects are produced, which severely damage the cell.

(*c*) There is an exact parallelism between the power of recovery shown by the metabolism and the viability of the tissues as shown by transplantation.

3. *Sodium Fluoride.*—This substance has been widely used in the study of inhibitions of cell metabolism. Most of the available data have been collected by Needham (1932), and reference here will only be made to work on tumour metabolism.

Dickens and Simer (1929) have made a thorough study of the checking of fluoride on anaerobic glycolysis of tumour tissue, but only one experiment is quoted of results found under aerobic conditions. A concentration of 5 millimoles was found to depress the respiration slightly, and check the aerobic glycolysis about 50 per cent.

Ewig (1929) states that M/1000 NaF slightly depresses the respiration and lowers the glycolysis (aerobic and anaerobic) 20 per cent. to 30 per cent. M/100 checks glycolysis to the extent of 90 per cent., with a concomitant fall of 30 per cent. to 40 per cent. in respiration.

In some of these experiments Ca-free Ringer-glucose-bicarbonate has been used. Although in a calcium-containing medium, the fluoride is partially precipitated, the results are substantially the same in the presence or absence of calcium. The general methods employed were as stated in the section on iodo-acetic acid.

Typical results for various concentrations are given in Tables III and IV, and fig. 6 gives the results of transplantation experiments.

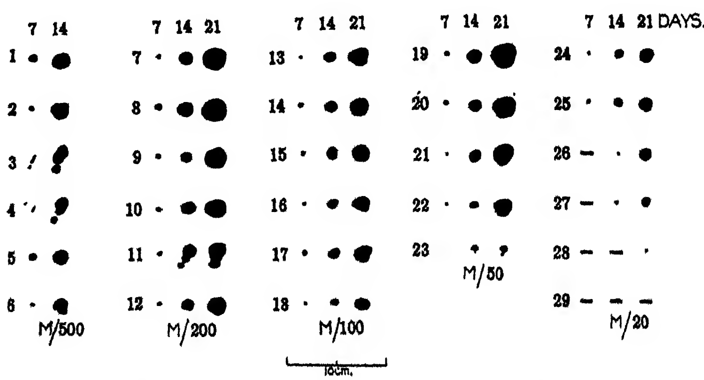


FIG. 6.—NaF in varying concentrations for 1 hour. NaF has a damaging effect on cancer cells, which is very pronounced with a concentration of M/20 and slight but still noticeable with a concentration of M/100. With M/200 there is complete recovery.

In figs. 7 and 8, the combined results of inhibition and recovery experiments are shown, effects on respiration and glycolysis being drawn separately.

The chief points of interest are as follows :—

(*a*) The character of the effects produced are similar to those found for iodo-acetic acid. Initially the depression of glycolysis is accompanied by a much smaller fall of respiration, but the value of the respiration falls progressively over the experimental period, and, with higher

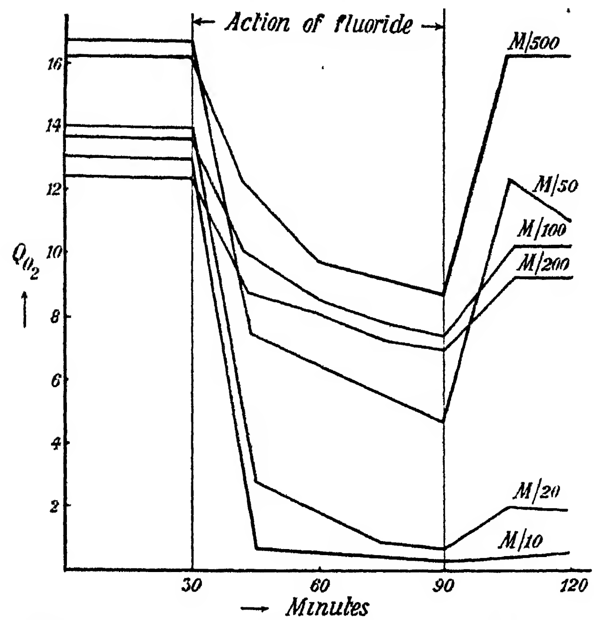


FIG. 7.—Effect of NaF on the respiration of tumour tissue, showing inhibition and subsequent recovery.

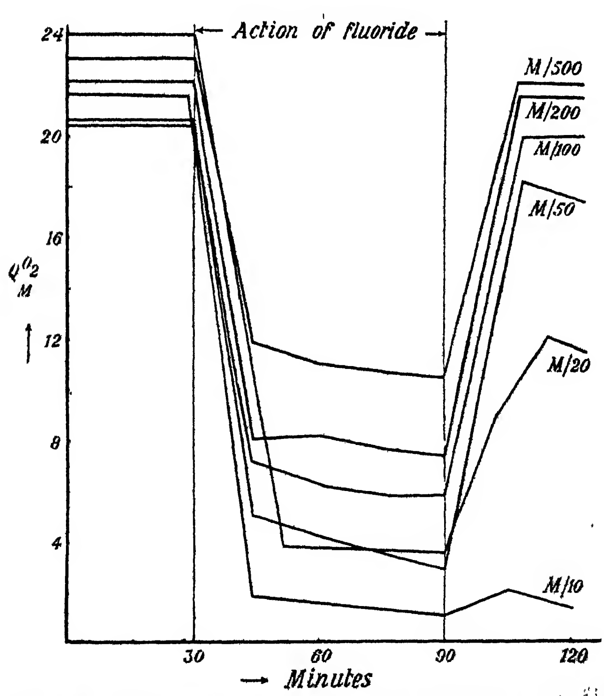


FIG. 8.—Effect of NaF on the aerobic glycolysis of tumour tissue, showing inhibition and subsequent recovery.

Table III.—Inhibiting Effects of Various Concentrations of Sodium Fluoride on Tumour Metabolism.

Tumour.	Medium.	Concentration of sodium fluoride.	Initial metabolism.			Metabolism after addition of sodium fluoride.				Per cent. fall in Q_{O_2}.	Per cent. fall in $Q_M^{O_2}$.	Per cent. fall in $Q_M^{N_2}$.
			Q_{O_2}.	$Q_M^{O_2}$.	$Q_M^{N_2}$.	Time.	Q_{O_2}.	$Q_M^{O_2}$.	$Q_M^{N_2}$.			
						min.						
J.R.S.	R.G.B.	M/500	18·5	23·7	—	15	13·0	11·5	—	30	52	—
						15	13·4	10·3	—	28	57	—
J.R.S.	Ca-free R.G.B.	M/500	16·2	23·0	—	10	12·4	11·7	—	23	49	—
						10	10·6	11·7	—	35	49	—
						10	9·7	11·2	—	40	51	—
J.R.S.	R.G.B.	M/200	12·7	22·7	33·4	15	8·5	8·0	7·2	33	65	79
						15	8·2	8·2	7·2	36	64	79
						15	7·8	6·6	6·8	38	70	80
J.R.S.	Ca-free R.G.B.	M/200	19·0	24·4	32·6	10	11·2	7·8	4·7	41	68	85
						10	6·3	4·7	3·2	67	81	90
						10	6·6	5·0	3·2	65	80	90
J.R.S.	Ca-free R.G.B.	M/200	13·8	19·6	—	15	10·1	4·8	—	27	76	—
						15	9·6	3·7	—	30	81	—
J.R.S.	R.G.B.	M/100	14·4	17·7	36·7	15	9·7	5·1	5·2	33	71	86
						15	9·7	4·4	3·3	33	75	91
J.R.S.	Ca-free R.G.B.	M/100	14·8	20·6	38·8	15	9·2	6·8	4·5	38	67	88
						15	9·2	5·8	3·6	38	72	91
J.R.S.	R.G.B.	M/50	17·0	21·7	—	10	7·6	4·7	—	55	78	—
						10	7·2	3·3	—	58	85	—
						10	5·2	1·6	—	70	93	—
J.R.S.	R.G.B.	M/20	7·7	23·8	30·4	10	1·8	3·8	6·2	76	84	80
						10	1·5	3·5	3·1	80	85	90
						10	1·6	3·6	3·1	79	85	90

concentrations of fluoride, tends to approach the value of glycolytic checking.

(*b*) The concentrations of sodium fluoride necessary to produce checkings comparable to those given by iodo-acetic acid, are very much larger.

(*c*) With concentrations of M/100 or less, there is almost complete recovery after 1 hour of treatment. M/50 allows of a large recovery, though degeneration is noticeable, and concentrations of M/20 or higher inflict definite damage on the whole cell, with only a small recovery of metabolism.

(*d*) The power of recovery of metabolism again runs parallel to the general viability of the cell, as demonstrated by transplantation experiments.

Table IV.—Experiments showing Recovery of Metabolism of Tumour Tissues kept in varying Concentrations of Sodium Fluoride.

Tumour.	Time of inhibition.	Concentration of sodium fluoride.	Initial metabolism.			Recovery metabolism.				Per cent. recovery of Q_{O_2}.	Per cent. recovery of $Q_M^{O_2}$.	Per cent. recovery of $Q_M^{N_2}$.
			Q_{O_2}.	$Q_M^{O_2}$.	$Q_M^{N_2}$.	Time.	Q_{O_2}.	$Q_M^{O_2}$.	$Q_M^{N_2}$.			
J.R.S.	60	M/500	11·9	19·3	32·6	min. 30	12·2	17·3	31·0	100	90	95
J.R.S.	60	M/200	14·8	21·3	36·2	30	11·9	20·0	37·0	80	94	100
J.R.S.	60	M/100	6·3	16·5	28·2	30	5·1	16·1	22·9	81	97	81
J.R.S.	60	M/50	12·4	18·3	30·2	15 15	10·2 8·6	16·3 14·1	26·2 24·1	82 70	89 77	87 80
J.R.S.	60	M/20	12·4	18·9	34·1	10 10 10 10	0 1·2 1·1 1·2	5·5 5·8 9·6 7·2	6·8 9·8 12·3 12·9	— 10 9 10	29 31 51 38	20 29 36 38
J.R.S.	60	M/10	12·4	18·9	34·1	10 10 10 10	1·2 0 1·5 0	2·0 2·6 3·3 3·3	4·2 5·6 7·3 5·4	10 0 12 0	11 14 17 17	12 16 21 16

Summary.

The limiting concentrations of well-known inhibitors of metabolism, which permit of subsequent recovery of tumour cells, have been investigated.

Hydrocyanic acid produces primarily an inhibition of respiration which is of the order of 85 per cent. to 95 per cent. in concentrations from M/1000 to M/500. As a secondary effect of this inhibition of respiration, the aerobic glycolysis of tumour tissue increases to a value approaching that of anaerobic glycolysis. The effect of hydrocyanic acid is completely reversible in all concentrations up to M/20.

The primary effect of sodium fluoride and iodo-acetic acid is an inhibition of aerobic glycolysis, which increases progressively with increasing concentration. This direct checking of aerobic glycolysis is accompanied by a smaller indirect inhibition of respiration which increases with the time of exposure.

Within certain characteristic concentrations these inhibitions are reversible after an experimental period of 1 hour. If these limits of concentration be exceeded, these two substances produce an irreversible damage to tumour cells. This damaging action appears even with concentrations which are insufficient to effect a complete or almost complete inhibition of glycolysis.

There is a close parallelism between the degree of metabolic recovery and the effect on the viability of the cell as measured by subsequent transplantations.

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The Action of Radium on Cancer Cells. II.—Some Factors Determining the Susceptibility of Cancer Cells to Radium.

By H. G. Crabtree and W. Cramer.

(Communicated by J. A. Murray, F.R.S.—Received May 5, 1933.)

The susceptibility to radium of different types of cells and tissues is one of the outstanding problems of radiotherapy, both from the theoretical and practical points of view.

In the organism, normal and malignant tissues exhibit great variations in their response to short-wave radiation. Some normal tissues, *e.g.*, lymph glands and testes, are extremely sensitive, while others are much less so. There are similar variations in sensitivity between different malignant new growths when irradiated in the organism. There is, however, more uniformity in the effects of irradiation on normal and malignant tissues when isolated cell-masses are studied. Similar biological and morphological changes are observed after irradiation of tissue cultures, whether of normal fibroblasts or tumour cells; and the metabolic disturbances induced in slices of tumour tissue, testes and spleen are of the same type and magnitude (Crabtree, 1932).

This parallelism of behaviour of normal and malignant tissues, both inside and outside the body, emphasizes the fact that there is nothing peculiar in the make-up of the cancer cell, which renders it specifically more vulnerable to radiation than normal cells.

Susceptibility to radiation would seem to depend, therefore, on some factor of vital importance, which is common to both normal and malignant cells.

It is known, moreover, that a given type of cell exhibits different degrees of susceptibility in different phases. Thus it has been found that a cell is most sensitive during the stage immediately preceding mitosis. It would follow that the common factor involved is capable of different degrees of manifestation in different types of cells, and in the same type of cell at different stages.

Of the known molecular systems found in cells the respiratory system is at once the most labile and the most vital. Its exact constitution is not finally settled, and its structural relationships are quite undefined. For the problem dealt with in this paper, the contending theories of the mode of assimilation of oxygen by a cell, are of little concern. All theories involve the idea that iron, in complex combination, plays a predominant part in catalytically promoting vital oxidations. Its mode of action is essentially that of a rapid alternation in valency.

The literature contains little evidence that the respiratory system is more vulnerable to short-wave radiations than other chemical mechanisms in cells. Indeed, some workers claim that X-rays have no effect on respiration, *e.g.*, Wels (1924) working with normal cells irradiated *in vivo*. Crabtree (1932), however, found that the damage done to cells irradiated continuously *in vitro* was manifested by a progressive degeneration in the respiration, while aerobic glycolysis remained comparatively constant.

These considerations suggested as a working hypothesis that the damage inflicted on the cell by irradiation operates through an action on its respiratory mechanism. If this were so, it should be possible to vary the susceptibility of cells to radium at will, by varying the functional activity of the respiratory mechanism. This can be tested experimentally by irradiating sections of transplantable tumours *in vitro* under different conditions, and then transplanting them. The results obtained on transplantation, *i.e.*, the number of positive inoculations and the rate of growth of the tumours found, give a measure of the susceptibility to radium of the cells under investigation.

The metabolism of tumour cells has been altered by various devices, some involving an interference with the respiratory system, and, for comparative purposes, others in which the glycolytic mechanism has been primarily affected :—

(1) Anærobiosis which prevents respiratory function by oxygen exclusion.

(2) The action of hydrocyanic acid which almost completely inhibits respiration in concentrations of M/1000 or more.

(3) Low temperature which, of course, retards all cell processes.

(4) The action of iodo-acetic acid which, in the concentrations used, effects a large inhibition of aerobic glycolysis together with a smaller indirect inhibition of respiration.

(5) The action of sodium fluoride which acts similarly to iodo-acetic acid, when used in larger concentrations.

Under all the experimental conditions used it had been shown by preliminary experiments that the viability of the cancer cell was unaffected, since on return to a normal environment its metabolism was restored and transplantation was always successful in 100 per cent., the resulting tumours exhibiting the normal rate of growth.

The general plan of the experiments was as follows: Thin sections of a transplantable mouse carcinoma were prepared as for the measurement of carbohydrate metabolism by Warburg's method. The strain 2146 was used exclusively in these experiments. The same strain had been used recently by us involving irradiation both *in vitro* and *in vivo* so that we were familiar with its behaviour (Cramer, 1932). It is a rapidly growing tumour which on inoculation takes in 100 per cent. One section was irradiated at 37·5° C. under standard conditions, which will be detailed presently. A second section was irradiated under standard conditions modified in one of the ways mentioned above. A third section was kept under these same modified standard conditions but not irradiated. At the end of the experiment the sections were inoculated into groups of from 6 to 8 mice, care being taken to inoculate pieces of equal size. The resulting tumours were charted in the usual way. The tumours obtained from the third section served as a control indicating that the cells had not suffered any damage under the experimental conditions and provided a measure of the normal rate of growth of the cancer cells. For the success of the experiments it is essential to observe strictly aseptic precautions.

Details of Experimental Technique.

Thin sections of tumour tissue, similar to those used for manometric determination of metabolism, were prepared under sterile conditions. Tumours from 10 to 16 days old were chosen, and only those practically free from necrosis were used. It is important to use sections of the same thickness.

The method of irradiation has been described in detail previously (Crabtree, 1932). The two applicators available were 23 mm. square, each containing approximately 110 mg. $RaBr_2 . 2H_2O$. The lids of the applicators were of silver 0·12 mm. thick, and a standard extra screen of 0·06 mm. was used throughout. The radiation therefore consisted of hard β-rays and γ-rays.

Preliminary experiments had shown that the two applicators were equally effective biologically, since equal times of exposure of tumour slices prepared from the same tumour produced the same results in subsequent transplantations.

Small mica-bottomed (0·04 mm. thick) cylindrical glass cells of capacity about 3 c.c. and diameter 1·2 cm. were placed centrally on the applicator.

The slice of tissue lay flat against the mica bottom of the cell, and was immersed in a Ringer-glucose-bicarbonate (R.G.B.) medium, introduced by Warburg (1926), either alone or modified by the addition of hydrocyanic acid, sodium fluoride, or iodo-acetic acid in suitable concentrations.

The cell and applicator were enclosed in a glass vessel, through which a rapid current of 95 per cent. $O_2 + 5$ per cent. CO_2 (for aerobic experiments), or 95 per cent. $N_2 + 5$ per cent. CO_2 (for anaerobic experiments) was passed, the whole being immersed in a thermostat at the appropriate temperature—normally 37·8° C. Before the addition of the tissue slices the R.G.B. medium and the containing vessels had been brought to the temperature at which irradiation was to be carried out.

This experimental technique is much more elaborate than that originally introduced by Wedd and Russ (1912) for irradiation *in vitro* and used subsequently with slight modifications by all other investigators. In that technique small pieces of tumour are placed with Ringer solution, covered and irradiated at room temperature. This method introduces a number of factors, such as absence of glucose and bicarbonate from the Ringer solution, difference of temperature between irradiation *in vitro* and *in vivo*, an inadequate supply of oxygen, etc., which depart from physiological conditions and thus increase the difficulty of interpretation. In selecting our standard conditions we were guided by the consideration that, if the activity of the respiratory mechanism was

concerned, as we suspected, in the reaction of cells to radiations, the standard conditions must be such as to enable respiration to proceed at a maximal and constant value during the period of the experiments. From observations on metabolism (Crabtree, 1932) it was known that the use of a Ringer solution without glucose and bicarbonate produced a rapid fall in the respiration and we have already published experiments showing that irradiation of sections placed in a simple Ringer solution produces a greater damage than is found when they are immersed in Ringer-glucose-bicarbonate solution at the same temperature. The effect of the other factors mentioned above on respiration is obvious and their influence on the susceptibility to radiation will be apparent from the experiments published in this paper.

Experimental Results.

When sections of the carcinoma strain 2146 are kept under these standard conditions for periods of 1 hour and then inoculated into normal mice, tumours develop in 100 per cent. and grow as rapidly as in an ordinary transplantation. Anaerobiosis, variations of temperature from 0° to 37·5° C., the presence of hydrocyanic acid, iodo-acetic acid, and sodium fluoride in the concentrations specified, have, as already stated, no effect, under these standard conditions, on the number of " takes " or the rate at which the tumours grow. The normal growth rate of this strain is such that at the end of the first week the tumours have reached a weight of 0·75 to 1·0 gram, at the end of the second week a weight of from 2·5 to 3·0 grams, and that after 3 weeks the tumours weigh from 6 to 8 grams, and threaten to break through the skin so that the mice have to be killed. The first effect of irradiating the sections is a retardation of growth without affecting the percentage of " takes." For example, exposure for half an hour to one of our applicators reduces as a rule the rate of growth to such an extent that the tumours are barely palpable at the end of the first week, and become visible only during the second week, and this delayed rate of growth persists for several weeks so that it is only at the end of the fourth or fifth week that the tumour begins to involve the skin. The percentage of successful inoculations may remain 100 per cent., but more often it falls to about 60 per cent. As the period of irradiation is prolonged to 45 minutes and 1 hour the rate of growth is progressively delayed, so that a tumour may become visible only in the third or even fourth week, and continue to grow very slowly. *Pari passu* with this diminished rate of growth there is a fall in the percentage of successful inoculations below 50 per cent. If two sections from the same tumour are irradiated

for the same period of time and inoculated, the results are approximately the same for the two sections.

But if work is continued with the same tumour strain continuously throughout the year, the strain occasionally passes into a phase where it is more radio-sensitive. In such a phase the retardation of the rate of growth is more pronounced and the number of successful inoculations is correspondingly diminished. Irradiation for 1 hour then completely prevents the subsequent formation of tumours, while exposure for half an hour reduces the percentage of successful inoculations to 50 per cent. or less. After a time the strain spontaneously recovers from this phase of increased radiosensitivity and reacts again to radium as described above. These alterations in the radiosensitivity are probably another manifestation of the cyclical changes in the biological behaviour of the cells of this tumour strain, which was studied in detail by Bashford, Murray and Cramer many years ago. These variations do not occur in sections from one and the same tumour.

The results of anaerobiosis, variations of temperature, and presence of hydrocyanic acid, iodo-acetic acid and sodium fluoride can be seen at once

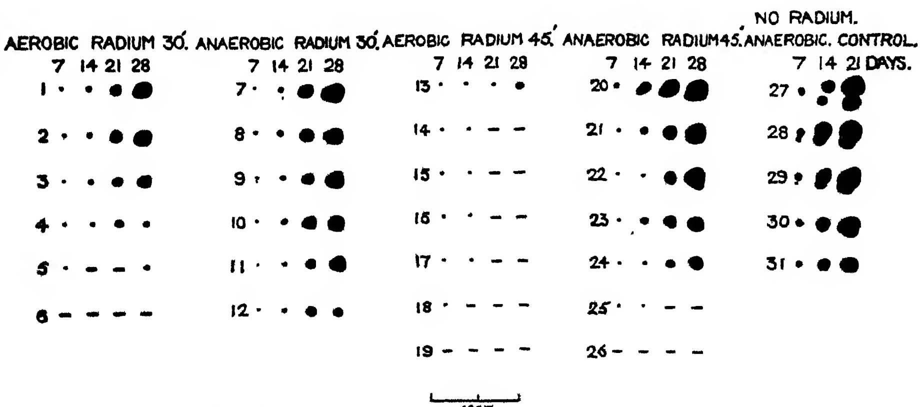

FIG. 1.—*Experiment* 167K. Anaerobiosis greatly diminishes the sensitivity of cancer cells to radium. Radium 30 and 45 minutes.

from the accompanying figures, in which the contour silhouettes of each tumour are charted weekly along horizontal lines. Each number represents a mouse. A large number of experiments have been carried out, in which over 2000 mice were used, and the results obtained were very consistent. The figures reproduced in this paper represent typical results.

Effect of Anaerobiosis.—Fig. 1 shows that malignant cells when kept under anaerobic conditions at 37·8° C. develop a pronounced insensitivity to radium.

Effect of HCN.—Sections kept in a Ringer-glucose-bicarbonate solution containing hydrocyanic acid in a concentration of M/1000 become much more radiosensitive, fig. 2. Fig. 3 gives the results of an experiment which deviated from the usual experimental condition so far as the sections were kept in the

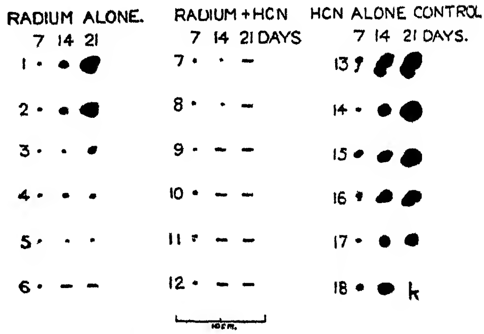

FIG. 2.—*Experiment* 166D. HCN in a concentration of M/1000 greatly increases the sensitivity to radium. Radium 30 minutes.

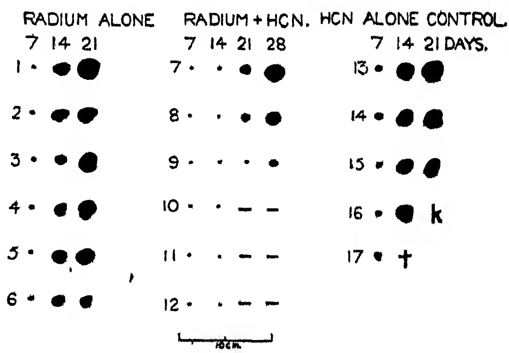

FIG. 3.—*Experiment* 164D. Shows the same effect as Experiment 166D with a smaller dose of radium, and more strikingly. An exposure to radium for 20 minutes, which by itself did not diminish the number of successful transplantations and only slightly reduced the rate of growth in the presence of HCN in a concentration of M/1000 diminished the number of successful transplantations by half, and very markedly reduced the rate of growth.

saline solution containing hydrocyanic acid for 40 minutes previous to irradiation. Radium was then applied for 20 minutes, the section remaining in the solution, so that the effect of hydrocyanic acid extended over a period of 60 minutes, while the application of radium lasted only 20 minutes. In spite of the relatively short duration of irradiation, the effect of radium was greatly intensified by the presence of hydrocyanic acid in the saline solution.

Effect of Temperature.—The effect is very pronounced, the action of radium being much greater on cells kept at low temperatures than on cells kept at higher temperatures, fig. 4. Cells kept at low temperatures are therefore more radiosensitive than the same cells kept at or slightly above body temperature.

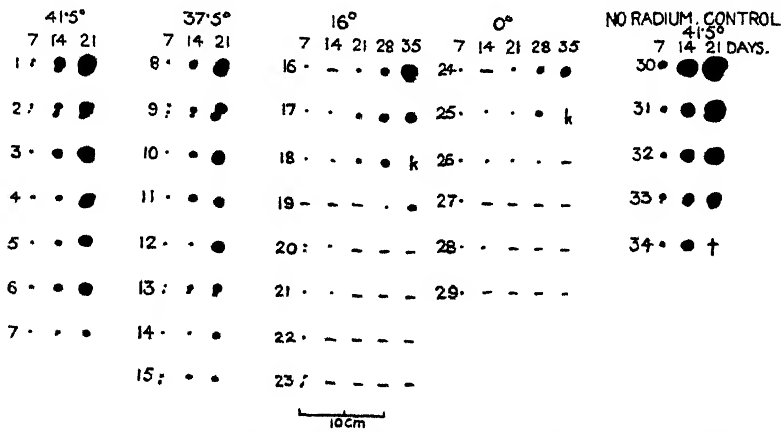

FIG. 4.—*Experiment* 170A. The sensitivity to radium increases as the temperature falls. Radium 30 minutes.

Comparison of Effect of Anaerobiosis with that of Hydrocyanic Acid.—This comparison was made in order to test the validity of the previous results. Since anaerobiosis diminishes the susceptibility of cells to radium and hydrocyanic acid increases it, when compared with cells irradiated under standard conditions, the difference should be intensified if cells irradiated under anaerobic conditions are compared with cells irradiated in the presence of hydrocyanic acid. Figs. 5 and 6 show that this expectation was fulfilled.

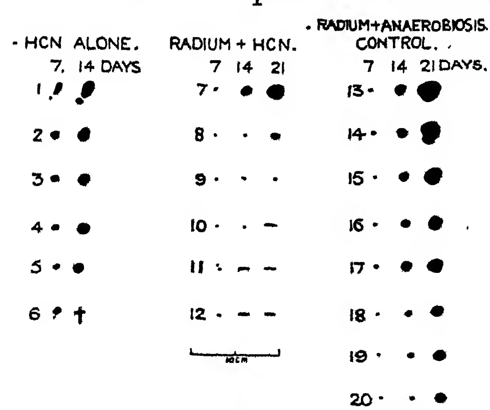

FIG. 5.—*Experiment* 169B. In this experiment the increased sensitivity to radium when respiration is checked by HCN, is compared with the diminished sensitivity to radium when respiration is checked by anaerobiosis. Radium 30 minutes.

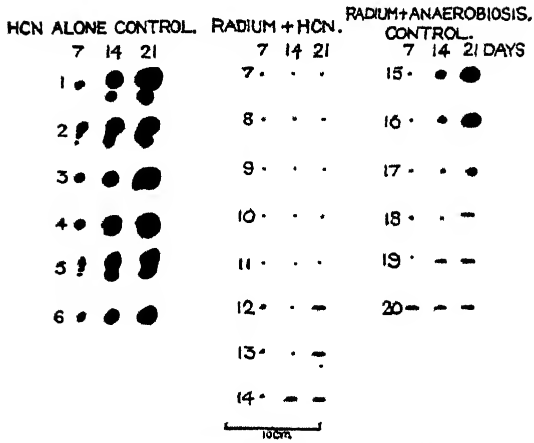

FIG. 6.—*Experiment* 169C. The same experiment as illustrated in fig. 5, but with exposure to radium of 45 minutes.

Effect of Iodo-Acetic Acid.—As is shown in Part I iodo-acetic acid primarily inhibits glycolysis in cells, though a fall of respiration, less in extent, accompanies this inhibition. Beyond a limiting concentration, approximately 3×10^{-4} M, irreversible damage is done to the cells, as shown by failure to recover their metabolism when placed in normal media and failure to give successful transplantations.

The experiments involving radium were therefore confined to concentrations in which the recovery of metabolism and growth of the cells were unaffected.

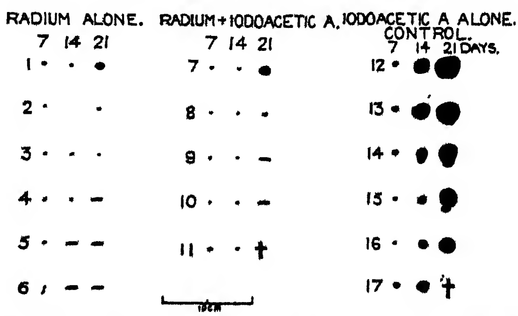

FIG. 7.—*Experiment* 162B. Iodo-acetic acid in a concentration of M/10000 has no effect on the sensitivity of cancer cells to radium. Radium 45 minutes.

Figs. 7 and 8 show that tumour cells thus treated show no marked difference in their susceptibility to radium from the control. This was the result obtained in four out of seven series of experiments. In one experiment there was an apparent slight increase in radio-resistance, while the opposite effect, a slightly increased radiosensitivity was observed in two series.

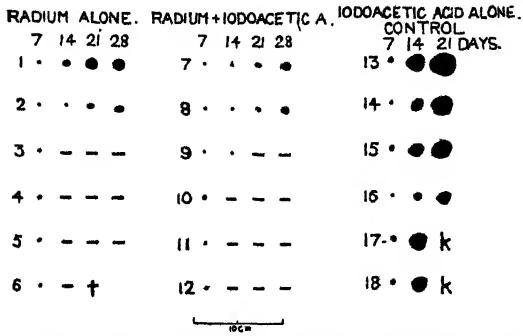

FIG. 8.—*Experiment* 161C. Same experiment as shown in fig. 7 but with a lower concentration of iodo-acetic acid (M/33000).

Effect of Sodium Fluoride.—As with iodo-acetic acid only concentrations were used which had been shown not to damage the cells, namely, M/200 and M/500.

With these concentrations and exposures to radium for 30 minutes and 45 minutes altogether 10 series of experiments were carried out. Of these six showed no definite effect of the fluoride on susceptibility to radium. Figs. 9 and 10 represent two such experiments. Two series showed a definite but

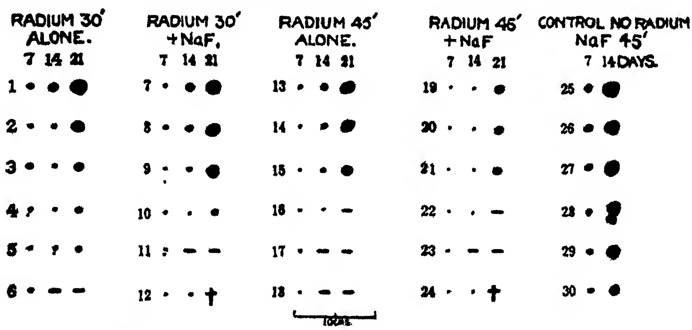

FIG. 9.—*Experiment* 176C. NaF in a concentration of M/200 has no effect on the sensitivity of cancer cells to radium. Radium 30 and 45 minutes.

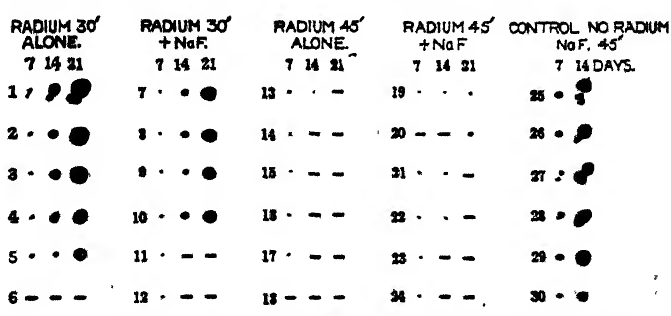

FIG. 10.—*Experiment* 176H. NaF in a concentration of M/500 has no effect on the sensitivity of cancer cells to radium. Radium 30 and 45 minutes.

slight increase, while another two series showed a slight but definite diminution in susceptibility to radiation.

Generally speaking, the effects produced by sodium fluoride closely resemble those observed with iodo-acetic acid and stand in striking contrast with those produced by anaerobiosis, hydrocyanic acid and cold.

General Discussion.

The experiments show that the susceptibility to the action of radium is not a fixed property of a given cell, but varies with its environment. Experimentally, the susceptibility of tumour cells can be varied *in vitro* within wide limits, by altering the functional activity of the respiratory mechanism. The standard conditions have been chosen so that the p_H, bicarbonate and glucose concentrations, and temperature approach those found physiologically, and that metabolism is maintained at a maximum throughout the experimental period.

When other factors are kept constant exposure to HCN or low temperature greatly increases the susceptibility to radium. Anaerobiosis produces the opposite effect, a greatly increased resistance to radium.

Both HCN and anaerobiosis produce an increase in glycolysis, which is of approximately the same order of magnitude. Nevertheless, the effect produced on radiosensitivity in these two conditions is in the opposite direction. This in itself indicates that the biological effect of radiations does not operate through an action on the glycolytic mechanism. This conclusion is confirmed by the fact that reagents such as iodo-acetic acid and sodium fluoride, which primarily inhibit the aerobic glycolysis of cells, have little or no effect in influencing the susceptibility of tumour tissue to the action of radium. The checking of respiration produced by these two reagents is a secondary effect, and as mentioned in Part I, is caused by conditions quite different from those responsible for the primary checking of respiration in anaerobiosis, in exposure to HCN and to cold. The experimental evidence strongly suggests that the functional activity and chemical condition of the respiratory mechanism with respect to its state of oxidation, reduction or combination, play a significant rôle in determining the after-effects of short-wave radiation on cells.

It would be irrational to imagine that this is the only chemical system in the cell affected by short-wave radiation, but its vital importance and lability

suggest that any irreversible damage done to it might well provide a starting-point for subsequent biological degeneration. It may also be pointed out that such a chemical conception is not at all incompatible with the view put forward by Vintemberger (1929) and by Holwerk and Lacassagne (1931) that the cell is not uniformly radiosensitive, but that the biological effects of radiations depend upon their action in certain circumscribed morphological areas.

These facts have an important bearing from the point of view of radiotherapy. It is known that tumours growing in badly vascularized tissues such as bone and cartilage are particularly radio-resistant. There is also the clinical experience that in patients suffering from anæmia the tumours do not as a rule react readily to radium. This has been confirmed experimentally by Mottram and Eidinow (1932) in rats with transplanted tumours which had been rendered anæmic by hæmorrhage. The phenomenon of radio-vaccination, in which a spontaneous growth treated with radiation insufficient to secure its regression becomes more radio-resistant to subsequent treatment, may perhaps be attributed to an interference with the blood supply resulting from the first treatment. What is probably the most important consideration arises from the fact that most neoplasms are badly vascularized and, if they are large, unequally vascularized. Different parts of a growth are therefore supplied with oxygen in a varying degree, so that they will vary in their susceptibility to a given dose of radiation. This would account for the clinical experience that in a tumour which has received an apparently equal dose of radiation some parts undergo regression, while others remain alive although their rate of growth may be temporarily delayed. Since a neoplasm is not necessarily homogeneous in its susceptibility to radium, it is clear that the problem of successful radiotherapy is not simply the physical one of producing a homogeneous field of radiation throughout the tumour, but that the biological conditions of the cells in different parts of the tumour have to be considered.

The results obtained *in vitro* with cold and hydrocyanic acid open up the possibility of increasing the susceptibility to radium of cancer cells *in vivo*, although the actual application of these agents is not a practical method in the living organism. But the fact that cancer cells are capable of being changed in the direction of increased susceptibility encourages the hope that an understanding of the process by which the various agents used in our experiments bring about the changes in the cells may make it possible to produce the same changes by other methods practicable *in vivo*.

Summary.

It is possible to produce experimentally great variations in the susceptibility to radium of cancer cells by acting on the respiratory mechanism of the cell. It is not possible to do so by acting on the glycolytic mechanism.

The devices used for acting on the respiratory mechanism were anaerobiosis, hydrocyanic acid and cold. Although all three conditions have the same general action of diminishing the functional activity of the respiratory mechanism, their effect on the susceptibility to radium is in opposite directions. Anaerobiosis diminishes, hydrocyanic acid and cold increase the susceptibility to radium.

The possible explanations of these results and their bearing on radiotherapy is discussed.

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Factors Influencing the Functional Development of the Male Gonad.

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(Communicated by F. H. A. Marshall, F.R.S.—Received May 8, 1933.)

[Plate 10.]

The recent advances in our knowledge of the function of the anterior pituitary lobe in relation to gonadic development and activity, have led to an investigation of the part played by the pituitary in controlling testicular growth and function. The understanding of the relation between the hypophysis and the gonads in the male is, in general, less advanced than for the female, in which this relation was first established.

It has been recognized that any action on the testis may be exerted on two morphologically and physiologically different parts: (1) The tissue concerned with the elaboration of ripe spermatozoa, and (2) the interstitial tissue producing the internal secretion responsible for the development of some of the secondary sex organs and characters. The development in these two tissues does not always follow a parallel course, and it appeared possible that different factors were responsible for controlling their growth and functional activity. Consequently a large number of investigations have been made on the action of various extracts and substances upon the growth and secretory activity of the testis. It is not here intended to review the whole literature on this subject, but a number of the effects obtained may be briefly recapitulated:—

(1) Numerous experiments have been recorded on the effect of specific hormonic factors. Following on the findings of Smith (1930), who showed that hypophysectomy resulted in atrophy of the testes and of the secondary sex organs, the action of various pituitary extracts was determined.

(i) *Pituitary Implantations.*—According to Smith and Engle (1927) implantation of anterior pituitary lobe (rat) caused an increase in the weight of the testis without any corresponding increase in the body-weight. On the other hand Biedl (1927) recorded that implantation of anterior lobe retarded the growth of the testis in both rats and mice. Borst, Döderlein and Gostimirovic (1930) claimed that implantation of anterior lobe increased the mytotic

divisions in the testis of the white mouse, and caused growth of the secondary sex organs.

(ii) *Pituitary Extracts.*—Evans and Simpson (1926), using an alkaline extract of anterior lobe, found a retardation of testicular growth. Biedl (1927) also agrees with this.

(iii) *Gonadotropic Extract from Pregnancy Urine.*—Though the effects of these substances in stimulating the growth of the secondary sex characters seem to be well established, there is a difference of opinion as to the influence on the testis itself. The findings of the majority of observers were, however, negative in that there was no effect on testicular growth, or spermatogenesis in rats and mice (Boeters (1931); Bourg (1931); De Jongh (1931), etc.)

(iv) *Œstrin.*—A number of investigators (Steinach and Kühn (1926), Lacqueur and De Jongh (1928), etc.) have shown that œstrin tends to inhibit growth and secretion of the testis. But Allanson (1931), using a pure sample of œstrin, was unable to obtain any interference whatsoever.

(v) *Male Hormone.*—Moore and Price (1932) have found that injection of male hormone into young rats causes inhibition of testicular development. The appearance of spermatogenesis was retarded, but there was some stimulation of the growth of the secondary sex organs.

(vi) *Suprarenal Extract.*—Using a Swingle and Pfiffner's extract, Corey and Britton (1931) claim to have accelerated spermatogenesis in rats. Cleghorn (1932), with similar material, was unable to obtain any effect.

(2) In addition to these specific hormonic factors, Moore and Price (1932) have, as a control experiment, injected various lipoid extracts (heart-muscle, liver, brain, etc.). They describe interference with the germinal epithelium of the testis, though the secondary sex organs showed no deviation from the normal.

In the experiments to be described in this paper an attempt has been made to investigate the factors influencing the morphological changes and secretory activity of the testis. The main object of the investigation was to elucidate those changes dependent upon known hormonic factors, and to differentiate them from any non-specific effects resulting from impurities which are often present in hormonic preparations. The experiments have been carried out on such a scale as to make any variations observed statistically significant.

Methods.

The experiments were performed on 317 rats. Only white rats were used. These belonged originally to the Wistar Stock and have been inbred for the

last three years at this Institute. The animals were kept on the usual stock diet; and precautions were taken to eliminate the effects of crowding by having the same number of experimental animals and controls in one cage. In all experiments litter mates were used as controls.

The following substances and extracts were used:—

(1) *Bovine Anterior Pituitary Lobes.*—These were obtained from a slaughterhouse and kept in a frozen condition until needed. Fresh batches were obtained weekly. The tissue was minced up immediately before injection by forcing through a fine sieve.

(2) *Ammonia Extract of Bovine Pituitary Lobe.*—This was prepared as follows. Ten grams of minced anterior lobe were stirred for three hours with 30 c.c. 20% ammonia (S.G. = 0·95). Most of the ammonia was then removed by passing through a current of air, and the whole then neutralized with glacial acetic acid. The solid substance was removed by centrifuging and an antiseptic, quinanil, added to the supernatant fluid in a concentration of 0·25%.

(3) *Œstrin Free Gonadotropic Preparation from the Urine of Pregnant Women.*—Prepared according to the method of Wiesner and Marshall (1932). The extract contained approximately five luteinizing mouse units per milligram.

(4A) *Adrenaline free Suprarenal Cortical Tissue.*—This tissue was obtained in the frozen state from the slaughterhouse, and the cortex roughly separated from the medulla. The cortical tissue was then minced up, and the adrenaline was removed by means of permutite. The efficiency of the adrenaline removal was in all cases tested by the action of the substances on isolated preparations of the small intestine.

(4B) A lipoid extract of whole suprarenal tissue, prepared in exactly the same way as the corpus luteum extract (Robson and Illingworth, 1931). No special precautions were taken to remove the adrenaline. (1 c.c. = 22 gm. whole gland tissue.)

(5) *Adrenaline-free Water-soluble Extract of the Suprarenal Cortex* prepared according to the method of Swingle and Pfiffner (1931) (1 c.c. = 20 gm. whole suprarenal). The hormonic content of this extract was not tested on adrenalectomized animals.

(6) *Rabbits' Heart-Muscle.*—The heart was removed immediately after the death of the animal and minced up in the same way as anterior lobe tissue.

In order to prevent sepsis an antiseptic (quinanil) was added to any implanted tissue, viz., heart, and anterior lobe. As has been previously shown, this

antiseptic does not appreciably affect the activity of gonadotropic hormones (Robson, 1931).

Results.

The experiments may be grouped conveniently under three headings :—

(1) Those in which anterior pituitary lobe substance or extracts were used.

(2) Those involving the injection of gonadotropic preparations from pregnancy urine.

(3) Those in which other substances, viz., suprarenal tissue and extracts, and muscle, were implanted or injected.

Experiments with Anterior Pituitary Lobe.

Anterior pituitary lobe substance was implanted into five groups comprising altogether 71 male animals, 74 litter mates serving as controls. The discrepancy between these two figures is due to the fact that three of the experimental animals died during the course of the injections. The rats received 0·05 c.c. of anterior pituitary substance every second day, together with a similar volume of 0·1% solution of quinanil in Ringer-Locke. All animals under four weeks old received only half these amounts. As will be seen in Table I, the periods over which the implantations were spread varied in the different groups (Group V is included in Table IV). At the end of the experiment the animals were killed by coal gas and weighed; the various organs were also weighed and subjected to histological examination.

Table I.—To show effects of anterior pituitary lobe tissue implantations.

Type	Exp.	Con.	Exp.	Con.	Exp.	Con.	Exp.	Con.
Number of animals	21	21	8	9	15	16	19	21
Age at beginning (weeks)	4	4	3	3	3	3	4	4
Age at end	6	6	6	6	5	5	10	10
Average body-weight (gm.)	91·7	88·0	73·2	77·4	43·5	42·7	85·0	78·8
Average weight of testis (gm.)	0·716	0·889	0·439	0·559	0·282	0·254	0·488	1·038
Average suprarenal weight (mg.)	22	17	20	13	14·5	10	25	17
Standard error wt. of testes (gm.)	0·033	0·052	0·043	0·068	0·025	0·023	0·062	0·105
Difference weight of testes (gm.)	0·173	—	0·120	—	0·028	—	0·550	—
Standard error of difference (testis) (gm.)	0·061	—	0·080	—	0·034	—	0·122	—

Implantation of anterior pituitary lobe always causes a marked increase in the weight of the suprarenal glands. On the other hand, there appears to be an inhibition in the growth of the testes. Moreover, this inhibition on

Fig. 1.—Microphotograph of testis. Animal (Litter C, weight = 97 gm., Table II) implanted with anterior lobe tissue. × 270.

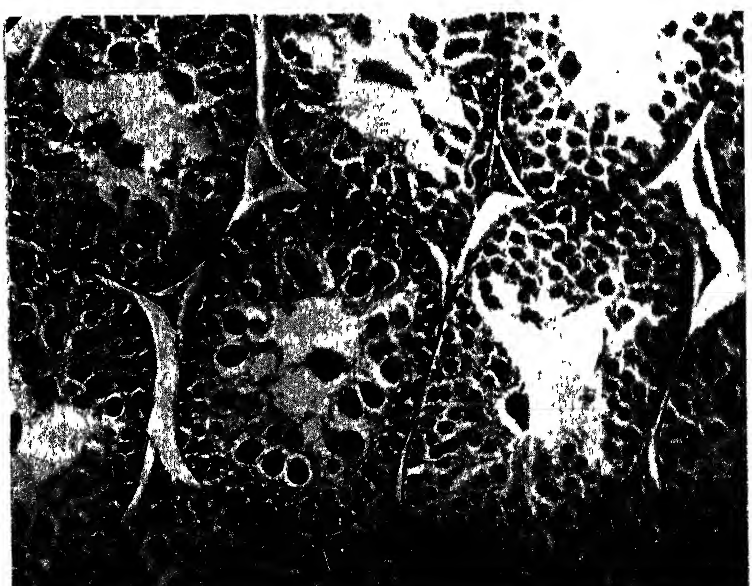

Fig. 2.—Microphotograph of testis. Litter mate control of animal in fig. 1. (Litter C, weight = 93 gm.) × 270.

(*Facing p.* 255.)

the whole increases with the period over which the injections are spread; for the effects in those animals implanted for six weeks were much more marked than those treated for shorter periods. It must, further, be emphasized that the treatment had little or no effect on the general health of the animals, as is evident from their body weight which is similar to that of the control groups. It may thus be concluded that any growth hormone that may have been present in the injected material did not appreciably influence the growth of the animals.

The vesiculæ seminales were carefully measured in the above experiments; the length taken being first in the coiled up state, and secondly after uncoiling. There was, however, no significant difference between the length of the vesicles in the treated animals, and in the controls. Histological examination of the testes revealed that the treatment had resulted in a definite effect, namely, inhibition of spermatogenesis. This was only slight in those groups treated over a short period, but became quite marked when the implantations were spread over a longer time interval.

Table II gives the full details for group (4). The estimate of the degree of spermatogenesis has been made on the following basis: S++ indicates that the great majority of tubules showed active full spermatogenesis in those testicles. In S+ less than half the tubules showed spermatogenesis, whilst S indicates that full spermatogenic activity was observed only in a few scattered tubules. The diameter of the tubules was carefully measured in the testes of both groups and was on the whole rather less in the implanted rats, than in the controls. No great differences can, however, be expected, as the diameter varies with the cube root of the weight. The additional observation was made that the degree of spermatogenesis roughly increases with the diameter of the tubules. In figs. 1 and 2, Plate 10, are typical microphotographs of injected animals and litter mate controls.

It is worth pointing out that quite apart from any effects of the injections, there exists a distinct correlation between size of testes and degree of spermatogenesis. This is well brought out in Table III, from which it can be seen that there is no evidence of spermatogenesis until the male gonads have attained a definite size. This appears to hold both in treated and in untreated animals, the implantations of pituitary resulting in an inhibition of growth and thus postponing spermatogenesis. This is in accordance with the above mentioned observation that spermatogenesis increases with the size of the tubules.

A further experiment was performed using an alkaline extract of anterior lobe. The animals were divided into three groups. The first group

Table II.—To show the effect on weight of testes and on spermatogenesis of implantations of anterior pituitary lobe tissue for a period of six weeks.

Litter.	Experimental animals.			Controls.		
	Weight of animal.	Weight of testes.	Spermatogenesis.	Weight of animal.	Weight of testes.	Spermatogenesis.
	gm.	gm.		gm.	gm.	
A	93	1·034	S++	83	1·258	S++
B	91	0·866	S+	98	1·274	S++
C	97	0·915	0	93	1·675	S++
	82	0·349	0			
D	74	0·293	0	76	0·939	0
E	46	0·199	0	50	0·276	0
				58	0·333	0
F	55	0·176	0	69	1·206	S++
G	65	0·238	0	69	0·972	S+
				87	1·081	S++
H	114	0·766	S+	113	1·964	S+
H	107	0·319	0	110	1·901	S++
I	76	0·715	S	67	0·816	S+
I	81	0·394	0	68	0·719	S++
I	69	0·209	0	66	1·010	S+
J	95	0·256	0	100	1·130	S++
J	105	0·608	0	94	1·380	S++
K	112	0·769	0	96	1·614	0
L	66	0·225	0	63	0·367	0
				59	0·542	S
Average	85·0	0·488	—	78·8	1·038	—

(9 animals) received 0·2 c.c. of the extract every second day for the first week, and 0·4 c.c. subsequently (1 c.c. of the extract—0·3 gm. of pituitary). The second group (8 animals) was implanted with the usual amounts of anterior lobe as described above, while the third group served as controls (two of which died during the course of the experiment).

The results are summarized in Table IV, which shows that the injection of the alkaline extract depresses the growth of the testes, though to a lesser extent than do the implantations. The slight increase in the weights of the suprarenal glands is not significant.

Table III.—Comparison of weight of testes with spermatogenesis in both implanted and control animals—six weeks implants.

Experimental.		Controls.	
Weight of testes.	Spermatogenesis.	Weight of testes.	Spermatogenesis.
1·034	S++	1·964	S+
0·915	0	1·901	S++
0·866	S+	1·675	S++
0·769	0	1·614	0
0·766	S+	1·380	S++
0·715	S	1·274	S++
0·608	0	1·258	S++
0·489	0	1·206	S++
0·443	0	1·130	S++
0·394	0	1·081	S++
0·349	0	1·010	S+
0·319	0	0·972	S+
0·293	0	0·939	0
0·256	0	0·884	S+
0·238	0	0·816	S+
0·225	0	0·719	S++
0·209	0	0·542	S
0·199	0	0·451	0
0·176	0	0·367	0
		0·333	0
		0·276	0

Table IV.—Comparison between the effect of pituitary extract and tissue.

Type of injection :	Pituitary extract.	Pituitary tissue.	Controls.
Number of animals	9	8	7
Age at beginning (weeks)	3	3	3
Age at end (weeks)	6	6	6
Average body-weight (gm.)	67·0	72·5	71·7
Average weight of testes (gm.)	0·512	0·462	0·667
Average suprarenal weight (mg.)	13	24	12
Standard error wt. of testes (gm.)	0·045	0·058	0·105

Experiments with Gonadotropic Preparations for Pregnancy Urine.

The experiment was performed on 19 animals comprising 9 injected rats and 10 litter mate controls. The rats which were about $3\frac{1}{2}$ weeks old at the beginning of the experiment, received 50 mouse units of the extract (in Ringer-Locke solution) every day over a period of 15 days. In the last 2 days only half that dose was given. The results are tabulated in Table V. In the first place, it will be seen seen that the treatment did not adversely affect the health of the

Table V.—To show the effects of injection of gonadotropic extract from pregnancy urine.

	Experimental.	Controls.
Age at beginning (weeks)	5	5
Age at end (weeks)	7	7
Average body-weight (gm.)	57·9	59·3
Average weight of testes (gm.)	0·887	0·408
Average suprarenal weight (mg.)	15	14
Average length—		
(a) Coiled vesicles (mm.)	15	5
(b) Full	23	7
Average weight of penis (gm.)	0·117	0·041
Average weight of genital tract less penis and testes (gm.)	0·715	0·085
Standard error weight of testes (gm.)	0·075	0·062
Difference in weights of testes (gm.)	0·479	—
Standard error of difference (testes) (gm.)	0·097	—
Number of animals	9	10

animals. There was, on the other hand, a marked effect on the development of the testes and on the whole genital system. In every case the testes of the experimental animals were heavier than those of the litter mate controls, and the average weight of testes in the injected animals is more than twice that of the control group. In spite of the increase in size there was no effect on spermatogenesis which was not more advanced in the injected group. Judging from a qualitative examination of the sections, the interstitial tissue would seem to be considerably increased.

As previously demonstrated by a number of investigators, the differences in the secondary sex organs are very marked. The seminal vesicles in the injected animals were enormously enlarged. The penis was also carefully measured and weighed. There is a definite effect on the length of the organ, but the differences are even more clearly shown by the weights, for the organs in the injected animals were nearly three time as heavy as those of the controls. At this stage of development, therefore, the male hormone evidently causes chiefly an increase in the diameter of the organ. It will, further, be noticed that the injections cause no significant alteration in the weight of the suprarenal glands.

Experiments with Suprarenal Extracts.

There are good reasons for believing that there exists a definite relationship between the cortex of the suprarenal and the gonads. This is chiefly supported by the data derived from clinical findings. The marked enlargement of the suprarenals observed in some of the experiments described above, was associated

with an inhibition of growth of the testes, and it was therefore decided to investigate in more detail the effects of suprarenal tissue and extracts on the development of the male gonads.

Experiments were performed with (1) adrenaline-free suprarenal cortical tissue, (2) a lipoid extract of the suprarenal gland, in the preparation of which no special precautions were taken to remove or destroy the adrenaline, (3) an adrenaline-free extract of the suprarenal gland, prepared according to method of Swingle and Pfiffner, 1 c.c. of extract = 20 gm. of whole gland tissue.

Implantations of Suprarenal Cortical Tissue.

Ten animals were implanted, 12 litter mates serving as controls. The rats were three weeks old at the first implantation, and received 0·05 gm. of cortical tissue together with an equal amount of 0·1% quinanil every second day for the first week. After that, for the next two weeks, double the amount was implanted. They were then killed after a total experimental period of three weeks; the results are tabulated in Table VI.

Table VI.—To show the effects of suprarenal tissue (adrenaline free) injections.

	Experimental.	Controls.
Number of animals	10	12
Age at beginning (weeks)	3	3
Age at end (weeks)	6	6
Average body-weight (gm.)	62·3	61·2
Average weight of testis (gm.)	0·242	0·385
Average suprarenal weight (mg.)	20	14
Standard error weight of testis (gm.)	0·088	0·078
Difference in weights of testes (gm.)	0·143	—
Standard error of difference (testes) (gm.)	0·118	—

The body-weights of the two series were quite similar, but on the other hand, the testes of the implanted animals were definitely smaller than those of the control group. The difference is comparatively small, but in view of the fact that we are dealing with litter mate controls it is very suggestive. Measurements of the vesiculæ showed no appreciable variation. The suprarenal glands in the experimental series were much larger than those in the control group. Histological examination of the material yielded no evidence of deviation from the normal; in both groups the great majority of animals showed no full spermatogenic activity.

Injection of Lipoid Extract of the Suprarenal Gland.

The experiments were performed on 11 rats, a similar number of litter mates serving as controls. The animals received 0·1 c.c. of the extract daily over a period of 20 days ,and were 6 weeks old at the end. The extract did not appear to be readily absorbed and most of the injected animals showed lumps at the sites of injection (but no sepsis). The results are given in Table VII.

Table VII.—To show the effects of injection of a lipoid extract of suprarenal glands.

	Experimental.	Controls.
Number of animals	11	11
Age at beginning (weeks)	3	3
Age at end (weeks)	6	6
Average body-weight (gm.)	40·8	56·8
Average weight of testis (gm.)	0·224	0·510
Average suprarenal weight (mg.)	15	12
Standard error body weight (gm.)	2·2	3·6
Standard error weight of testis (gm.)	0·024	0·063
Difference in weights of testes (gm.)	0·286	—
Standard error of difference (testes) (gm.)	0·067	—

It will be seen that there was a definite inhibition of growth, and also a marked effect on development of the testes. In fact, the average weight of the gonads in the experimental group was less than half that of the controlled group. This difference definitely cannot be accounted for by inequality in body-weight. At the end of the experiments, none of the injected animals showed any full spermatogenic activity, such as was observed in three of the control group. The suprarenal glands in the experimental group were definitely heavier than in the control animals.

Experiments with Swingle and Pfiffner Extracts.

Three different groups of animals were used comprising altogether 22 experimental rats and 17 litter mate controls. The age at the beginning of the experiment varied from 20 to 29 days.

The animals were injected daily for 25 days with 0·5 c.c. of the extract, corresponding to 10 gm. of suprarenal tissue. In all three groups the body-weight of the experimental animals at the end of the injection period was slightly less than that of their litter mates. The results obtained are summarized together in Table VIII. The average weight of testis was distinctly

less in the injected animals and this also applies for the separate groups. The results may also be stated rather differently: altogether 12 litters were used in this experiment, in 10 of these the weight of the testis of the experimental animals was smaller than in the controls, while in two litters (comprising altogether 4 animals), the converse was observed. Histological examination showed no marked difference between the two groups but suggested on the whole that some inhibition of spermatogenesis had occurred. There was no significant difference either in the weights of the suprarenal glands or in the lengths of the vesiculæ seminales in the two groups.

Table VIII.—To show the effects of suprarenal gland extract (Swingle and Pfiffner) injections.

	Experimental.	Control.
Number of animals	22	17
Age at beginning (days)	20–29	20–29
Age at end (days)	43–52	43–52
Average body weight (gm.)	65·7	75·3
Average weight of testis (gm.)	0·490	0·686
Average suprarenal weight (mg.)*	13·3	13·0
Standard error, weight of testis (gm.)	0·075	0·109
Standard error of difference (testes) (gm.)	0·132	—

* These figures refer to 16 experimental animals and 13 controls.

Heart Muscle.

So far all experiments described were performed with substances containing definite hormones and reported to affect the genital organs. Nevertheless, the possibility had to be considered that at least some of the results obtained were due to the admixture of proteins and other impurities, and were not of a specific character. A number of experiments was therefore performed in which heart muscle was implanted under similar conditions. Seventeen animals three weeks old were injected every second day with 0·025 gm. of heart muscle and a similar amount of 0·1 per cent. solution of quinanil for one week, and with twice these amounts of heart muscle and quinanil solution for a further 26 days. Table IX shows the result obtained.

The treatment caused an inhibition of growth; the development of the gonads was markedly affected, the inhibition being probably more than could be accounted for by the retardation in the growth process.

Further, there was also a marked effect on the seminal vesicles, the weight of which were appreciably less in the experimental group. The weights of the suprarenal glands were substantially the same in both groups.

Table IX.—To show the effects of implantations of rabbit's heart muscle.

	Experimental.	Controls.
Number of animals	17	17
Age of beginning (days)	25	25
Age at end (days)	58	58
Average body-weight (gm.)	61·5	74·3
Average weight of testis (gm.)	0·350	0·566
Average suprarenal weight (mg.)	17	16
Average weight of seminal vesicles (mg.)	12	20
Standard error body-weight (gm.)	2·2	2·6
Standard error weight of testis (gm.)	0·039	0·041
Standard error weight of seminal vesicles (mg.)	1·1	1·0
Difference in weights of testes (gm.)	0·216	—
Standard error of difference (testes) (gm.)	0·057	—

The fact that similar results were obtained with anterior pituitary and heart muscle suggested the need for a more accurate comparison of the effects of these two substances. Litters containing at least 3 animals were selected and used to make up three groups (of 8 animals each) with one litter mate in each group. The first group was implanted with anterior lobe, and the second group with heart muscle; in both experiments the animals received the same doses as previously described. The implantations were spread over a period of 41 days, and the animals were 24 days old at the beginning of the experiment. The data are given in Table X. The body weights did not differ significantly in the three groups, but the average for the animals implanted with heart muscle was rather less than that of the others. Both types of implantations affected the weights of the testes, but the action of the pituitary was much more marked than that of the heart muscle. It would therefore be expected that the remainder of the genital tract would be influenced in the same way, but this expectation was not realized, and actually the heart muscle inhibited the accessory sex organs to a greater extent that did the anterior pituitary.

The vesiculæ, prostates, vasa deferentia, and epididymes were weighed together; the weight of these organs was less than the controls in both groups, but was appreciably less in the heart muscle group than in the pituitary group. The weight of the penis was substantially the same in both implanted groups, but definitely less than in the controls.

Table X.—Comparing the effect of implantations of anterior pituitary tissue and heart muscle.

Type of implants:	Pituitary tissue.	Heart muscle.	Controls.
Number of animals	8	8	8
Age at beginning (weeks)	4	4	4
Age at end (weeks)	10	10	10
Average body-weight (gm.)	109	98	113
Average weight of testis (gm.)	0·760	1·113	1·290
Average suprarenal weight (mg.)	28	21	18
Average length seminal vesicles of—			
Coiled (mm.)	8·4	8·1	9·1
Full (mm.)	11·4	10·9	12·5
Average weight of penis (gm.)	0·084	0·082	0·104
Average weight of genital tract less penis and testes (gm.)	0·371	0·311	0·444
Standard error body-weight (gm.)	6·6	10·1	6·2
Standard error weight of testis (gm.)	0·094	0·176	0·160
Difference in weights of testes (gm.)	0·530	0·177	—
Standard error of difference (testes) (gm.)	0·185	0·238	—

Histology.

The pituitary implants caused the same effect as in the previous experiment, namely, inhibition of spermatogenesis. The heart muscle did not affect spermatogenic development.

The suprarenal glands were little affected in the heart muscle groups, but showed the usual hypertrophy in the pituitary group.

Discussion.

It seems to be well established that the pituitary controls the growth and development of the male gonads. Not only is there cessation of growth after hypophysectomy, but homo-implants of pituitary substance in rats can, to some extent, at least, replace the normal pituitary activity, causing regeneration of all testicular functions (Smith, 1930). Further, Smith and Engle (1927) claim that homo-implants in normal animals increase the weight of the testes as compared with controls, although the published data indicate only small differences in a limited number of animals. They further state that bovine pituitary substance over 8 hours after removal had no effect in hastening sexual maturity, even in female animals.

In contradistinction to these results we have obtained, by the implantations of bovine anterior lobe substance, a marked inhibition of development of the

testes. Not only was there failure in the growth, but also retardation of spermatogenic activity. The injection of an alkaline anterior lobe extract caused a lesser effect on testicular growth. A similar experiment on testicular size is reported by Biedl (1927) with pituitary implantations, and Evans and Simpson (1926) who injected alkaline extracts.

There appears to be no doubt, however, that an unspecific factor plays at least some part in the inhibition observed; for a similar effect could be obtained by a variety of other means such as the administrations of suprarenal preparations in various forms: (1) implantation of adrenaline-free cortical tissue, (2) lipoid extract containing adrenaline, and (3) adernaline-free extract prepared according to the method of Swingle and Pfiffner. Further, the implantation of rabbit heart muscle also produced some inhibition of testicular development. In fact, with the exception of a highly purified gonadotropic preparation from pregnancy urine, all substances implanted or injected, inhibited gonadic development.

That the effect observed involved not only the morphological structure of the gonads but also their secretory activity, is shown by measurements on the vesiculæ-seminales, and other secondary sex organs. Contrary to expectations, the most marked inhibitory effect on the secondary sex organs was obtained with the heart muscle. The degree of inhibition affecting the seminal vesicles, penis and other parts of the genital tracts, was such that the weights of these structures in the experimental animals were at least 30 per cent. less than the controls. These effects were less pronounced with anterior lobe and with suprarenal tissue, both of which produced a more marked effect on the testicular weight.

An analysis of the data obtained shows that in treated animals the modification of the size and secretory activity of the testes do not necessarily follow a parallel course. Thus, in some cases marked inhibition of growth of the testes was associated with little or no effect in the seminal vesicles; whilst in other experiments, a pronounced effect on the secondary sex organs was associated with a comparatively small reduction in the size of the testes. A similar type of result has been observed when adrenaline has been injected into immature male mice (Robson, 1932). This caused a marked inhibition in the growth of the seminal vesicles, though the weight of the testes in the injected animals was little less than that of the controls. These results suggest that the mechanisms which control the spermatogenic and the secretory structures in the testis are not completely identical and can be separately influenced.

The fact that anterior lobe implantation caused a marked change in size of the testes and inhibited spermatogenesis, with a comparatively small effect on the seminal vesicles, while such an unspecific stimulus as heart muscle chiefly involved the gonad secretory activity leaving spermatogenic development practically unaffected, suggests the possibility that the inhibitory action of the anterior lobe may be partly due to a specific factor which need not necessarily exert its action directly on the gonad. Such a conclusion, however, is at the present only tentative. In this connection, it is of interest to note that Evans and Simpson (1928) showed that the growth hormone exerted an antagonistic action on the pituitary sex hormone.

A number of experimental procedures which caused marked inhibition of growth of the testes, also resulted in a considerable hypertrophy of the suprarenal glands. This applies especially to implantations of anterior lobe and suprarenal cortical substance. An inhibitory effect on the growth of the testes was also brought about by extracts of the suprarenal gland without any significant alteration in the weight of the suprarenals of the injected animals. These data differ from the negative findings recently reported by Cleghorn (1932) and are diametrically opposite in type to the results described by Corey and Britton (1932). Clinical evidence suggests that hyperactivity of the suprarenal cortex occurs concomitantly with testicular hyper-function. The evidence outlined above appears to indicate, however, that any suprarenal hormonic factors present in the lipoid fraction are not responsible for such an action; in fact they appear to cause an inhibition. Further, the hypertrophy of the suprarenal brought about by the experimental means outlined above, may be a factor in the inhibition of the testes obtained in the same experiments.

Gonadotropic Preparation.

The experiments of various investigators with gonadotropic preparations of pregnancy urine reveal a divergence in their results. Both increase and decrease in the size of the testes have been obtained (De Jongh (1931); Boeters (1930); Neumann and Peter (1932), etc.) It is generally agreed that spermatogenic development is not stimulated. Our own experiments with a specially purified preparation showed a definite hypertrophy of the male gonads with no effect whatever on spermatogenesis. It is, therefore, evident that gonadotropic hormones may be involved in the control of growth of the testes, but that additional factors are essential for the development of spermatogenesis. Although the data obtained from normal animals and from those receiving

pituitary implantations show a general correspondence between the weight of the testis and the appearance of spermatogenesis, the figures derived from the experiments with gonadotropic hormone reveal that testicular size and spermatogenesis are not necessarily related.

As a result of a large number of experiments, Moore (1932) has elaborated an attractive explanation for the phenomena of hormone antagonism. He suggests that the pituitary secretion is controlled by œstrin, and the male hormone, both of which can depress its activity in either sex. His experiments with various types of injections seem to support this point of view. The validity of his results is based upon the assumption that the changes observed are caused by the specific hormonic factors, and indeed, in his control experiments with lipoid extracts of liver, brain and heart, the secondary sex organs remained unaffected, although he obtained in some experiments damage of the germinal epithelium. Several investigators have, like Moore, described interference with the testicular development by means of various œstrin preparations. Against this Allanson (1931) using a pure preparation of œstrin, failed to obtain any effect on the testis. This suggests the possibility that the results obtained by the other authors mentioned above may have been due to some impurity, possibly non-specific, in their preparations.

Our own experiments with heart muscle undoubtedly show that non-specific substances can interfere with growth of the testes and secretion. The conclusions of Moore on hormone antagonism cannot, therefore, be accepted as final until his results have been obtained with hormonic preparations free from impurities.

Summary.

(1) All experiments were performed on immature male white rats belonging to the Wistar stock. Implantations or injections were started when the animals were 3 to 4 weeks old and continued for several weeks. Litter mates were invariably used as controls.

(2) The implantation of bovine anterior pituitary lobe substance caused (i) inhibition of growth of the testes; (ii) inhibition of spermatogenic development; (iii) some inhibition of the growth of the secondary sex organs; (iv) hypertrophy of the suprarenal glands; (v) no effect on body weight.

(3) The injection of a highly purified extract prepared from the urine of pregnant women and containing gonadotropic hormones caused (i) increase in the weight of the testes; (ii) no effect on spermatogenesis.

(4) The implantation of adrenaline-free suprarenal cortical tissue or injection of suprarenal extracts (lipoid extract; Swingle and Pfiffner extract) caused

(i) inhibition of growth of the testes; (ii) a variable and usually small effect on the body-weight, insufficient to account for the effects on the testes.

(5) The implantation of rabbit's heart muscle brought about (i) some inhibition of growth of the testes; (ii) inhibition of the growth of the secondary sex organs; (iii) no effect on spermatogenesis. These effects have been carefully compared with those resulting from the implantation of bovine anterior lobe.

(6) The results are discussed in relation to the mechanism controlling development and function of the testes and to the problem of hormone antagonism.

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Discussion on Experimental Production of Malignant Tumours.

(June 15, 1933.)

Dr. J. A. Murray, F.R.S.: The investigations of the last 30 years have proved that the cells of the higher vertebrates, under appropriate conditions, are capable of unlimited proliferation, and it is of importance in discussing the problems of experimental carcinogenesis to remember this fact, and to have clear ideas of the characteristic features of the proliferation of new growths, including cancer. This is very necessary because nothing is commoner in the literature of the subject than loose statements that this or that agent confers on the cells powers of unlimited proliferation, which is nonsense, seeing they already possess these powers. The essential feature is the uncontrolled or autonomous character of the cellular proliferation, that is to say, the agencies which are effective in the body in limiting the rate and amount of growth and cell division are ineffective against true new growths. This is true of both classes of new growths, the benign as well as the malignant. Examples are seen in the fatty tumours, lipomata, which go on increasing in size in an emaciated individual and the uterine myomata which grow progressively even after the menopause while the ordinary uterine muscle is shrinking or quiescent. The malignant new growths, carcinoma and sarcoma, exhibit this feature still more clearly because many show a more rapid rate of growth. The other distinctive features of the malignant as contrasted with the benign new growths are differences in degree, rather than in kind, and their more perfect independence or autonomy, manifests itself in infiltrative progress, disorganizing and destroying the normal tissues encountered, by pressure from without, or occlusion and rupture of blood supply. The stretching and tearing of the walls of blood and lymph vessels opens the way for the entrance of smaller or larger aggregates of the parenchyma cells into the vessels and these, transferred to remote situations further exhibit their independence and adaptability to new surroundings, by the formation of secondary centres of growth, or metastases. It is these manifestations of neoplasia which render cancer so formidable a problem in treatment.

These new proliferative conditions arise in limited localized foci and once they have reached a size sufficient for recognition further increase takes place

only from the descendants of the already transformed cells without fresh accessions from the surrounding elements of the same kind. The original focus may be solitary, or several foci (all minute) may appear almost simultaneously, and soon fuse into one tumour. One type of cell only acquires these new properties and by its multiplication gives rise to the new formation, so that it is usually possible by microscopic examination to infer the tissue of origin, even after the tumour has reached a great size. The growths of any one tissue do not reproduce the full characters of the differentiated tissue of origin to the same extent. They form a continuous series ranging from apparently perfect reproduction of the histology of the parent tissue to a condition in which no specific differentiation can be recognized at all. Much unnecessary ink has been spilt in devising suitable words to describe this "undifferentiated," "dedifferentiated," "anaplastic" or "embryonic" state, but what is worth emphasizing is the fact that the degree to which it occurs is relatively fixed in any one new growth, and is maintained practically unaltered throughout its course. The rate of growth shows a similar uncorrelated series of gradations and there is much evidence to show that this also is (or at least may be), an initial, inherent, quality of the essential constituent parenchyma cells.

Many of the ideas and conclusions in the preceding summary have obtained a welcome precision and validity from the study of the transplantable malignant new growths of the mouse and rat during the past 30 years. In consequence we may now, with some confidence, define the new growths as single tissue proliferations arising in a localized area, growing from their own resources in an uncontrolled manner, showing a continuous graduated series in those arising from any one tissue, both in histological structure and in rate and habit of growth. In spite of this great range any one variety is relatively permanent. Descriptive convenience has determined the order in which these characters are enumerated as I find it impossible to give them marks indicating relative importance. We are still without a satisfactory explanation of the process which embraces all these characters and it is hazardous (to me impossible) to accept any hypothesis of the nature and origin of new growths which omits any one of them from its ambit.

The necessity, here indicated, of keeping in mind several nearly independent biological processes is common to many pathological problems. It prevents that artificial simplification of the material, which has been so powerful an instrument in the advancement of the physical sciences, and to a considerable extent of physiology.

The attempted solutions of the problem of the nature and causation of malignant new growths, carcinogenesis, which it will be profitable to discuss at this meeting fall into three groups :—

(1) The genetical hypothesis originating with Boveri, and modified by Bauer.

(2) The virus hypothesis particularly associated with the names of Borrel, Rous, and Gye.

(3) The special form of the chronic irritation theory of Virchow which has arisen on the basis of the work of Yamagiwa on the experimental production of tar cancer.

(1) Boveri's hypothesis was an outcome of his well-known studies on the development of disperm-fertilized echinoderm eggs. Boveri had shown that the haploid number of chromosomes or any multiple of it was compatible with normal development. He ascribed the pathological development of these disperm eggs to chromosome defect, one or more of the four primary blastomeres having received an incomplete set of chromosomes from the multipolar first segmentation division. Direct observation on cancer cells showed that a constant abnormal chromosome number was not found in any one new growth, and the hypothesis was accordingly modified by assuming an invisible, partial, damage to individual chromosomes. The necessity of assuming a haphazard origin from multipolar mitosis retreated into the background, Boveri's own attempts to initiate malignant new growths by this means having failed. It was an easy step to shift this assumed damage to the genes, the invisible chromosome constituents bearing the hereditary factors. This assumption made the hypothesis practically invulnerable by withdrawing it from the possibility of experimental proof or disproof. The main objection to it now is the necessity involved of assuming a large number of constituent units in each gene, to allow for the great number of slight modifications presented by the new growths of any one tissue, all practically permanent.

(2) *The Virus Hypothesis*.—The modern discussion of this hypothesis revolves around the study of the filterable tumours of the domestic fowl first discovered by Rous. Rous himself described four strains of sarcomata, all transmissible by cell-free filtrates, and many pathologically distinct. Subsequent workers, according to a recent review by Murphy, have brought the number up to 30. Their most remarkable feature is the biological and histological constancy of the growths propagated by cell-free filtrates and the

multiplication of the active constituent of the filtrates, during growth in the susceptible animal. While the latter character points to a living, self-reproducing agent, or virus, the variety of stable strains, each requiring a slightly different virus, has proved too much for many workers and an enzyme-like derivative of the cell protoplasm, has been imagined to surmount this fence. Gye's genial conception of a factor derived from the host cells and carrying all the specific distinctive characters of the tumour strain, acting in conjunction with a relatively non-specific virus, is still without direct experimental proof. Like the enzyme modification the greatest obstacle to its acceptance is the failure to demonstrate cell-free transmission in the laboratory strains of mouse and rat tumours. Till this is accomplished it is dangerous to transfer conceptions arising out of the study of the filterable new growths of the fowl, to the elucidation of the nature and causation of the new growths of other animals and man. On the other hand, the similarity in behaviour of the filterable tumours to the new growths of mammals imposes the necessity of investigating them, till the nature of the agent is elucidated and its mode of action, so different from that of the majority of viruses, is explained.

(3) The experimental confirmation of Virchow's chronic irritation theory in the last 20 years, has established its validity as a concise description of the emergence of cancer after prolonged, localized, slight, irritation of the tissues by a variety of agents, and nothing more. Wide differences of opinion still find expression as to how this result is brought about. As an important section of the present discussion will bear on the various aspects of this problem it may be of service to those without personal experience of the process to describe the course of a typical experiment of tar cancer induction in mice. If 100 mice be painted twice weekly on a small area of the back the following series of changes are observed. In the first two or three weeks the hair hypertrophies and becomes thicker and longer on the painted area. The hypertrophied hairs then fall out and are not replaced and the tarred area becomes hairless and remains so. If the skin at this stage be examined microscopically the covering epithelium is found to be thickened, the sub-epithelial tissues show a variable amount of increased cellularity and infiltration with lymphocytes, mast cells, and a few polymorph leucocytes. In the deeper layers, sometimes even below the panniculus carnosus, the tips of hypertrophied hair follicles are found, but these ultimately disappear as painting is continued. After 12 to 16 weeks localized thickenings of the squamous epithelium appear as discrete warty prominences in the otherwise smooth skin surface. One or more of these increase in size and project above the surface as warty out-

growths. Microscopically they have the structure of benign papillomata and if painting is now stopped they may either (1) disappear completely, (2) remain as warty papillomata for a long time, slowly increasing in size, or (3) after a variable length of time, begin to grow more rapidly, becoming adherent to the surrounding skin and deeper tissues. Their further progress is continuous and the growths manifest all the features of cancer both to the naked eye and microscopically. The death of the mouse follows in 6 to 8 weeks and after death metastases may be found in the nearest lymph nodes and in the lungs, occasionally in other internal organs. In a certain number of animals, the proliferative condition is malignant from its first appearance, without the apparently benign intermediate stage. We have not yet any method of distinguishing the papillomata which will remain quiescent from those destined to undergo the further progressive malignant development, and in consequence during the remainder of the time of observation more and more of the apparently benign warts pass into the third (malignant) group till after 12 to 15 months all the mice have developed tar cancers with the exception of a few which still bear simple warty papillomata and one or two in which no proliferative changes have occurred at all. The same succession of changes follows the intermittent long-continued application of a number of different chemical substances, some related to coal tar, others not. The processes involved are apparently the same as those responsible for the development of several forms of industrial cancer, and the experimental work has provided a secure basis for the incrimination of tar, shale oil, soot and arsenic in the causation of the corresponding industrial cancer. In addition, other chemical substances exuded by animal parasites, and physical agencies such as cold, ultra-violet light, X-rays and radium radiation can replace coal tar. The preliminary silent period and successively appearing growths as the applications are continued, are exhibited by the intentional and unintentional experiments alike. They furnish a simple, natural, explanation of the age incidence of cancer, a feature which formerly seemed mysterious.

While the constancy of the results of such experiments and the frequency and restriction of certain cancers to those following definite occupations involving exposure to the same substances fully justify the naming of them as *carcinogenic*, it does not take much reflection to see that the essential nature of cancer and the cellular changes involved, remain unexplained. Although the agents are applied uniformly over a considerable area, the cancerous process appears only in localized foci and not diffusely. Even in those cases in which a diffuse origin throughout a whole organ is encountered it is almost certainly due to

close juxtaposition of many minute foci and not to a spreading involvement of adjacent unaffected cells.

The chemical and physical carcinogenic agents, in my opinion, act indirectly; they set up conditions in the tissues such that, as a new departure, here and there cancerous foci are started. The chemical properties of these agents give no indication of how the autonomous, uncontrolled type of proliferation is induced, indeed they are so varied that it is difficult to see how they can give any definite indication of the nature of the cellular mechanism involved. Until this difficulty is at least partially obviated it seems unnecessary to indulge in the further speculation, that the less precisely known carcinogenic agents, secretions of animal parasites, application of cold, X-rays, etc., produce this effect by liberating in the tissues substances, more or less closely related chemically, to one or other of the pure substances with carcinogenic action.

From this brief summary it is clear that the experimental induction of malignant growth reproduces perfectly the phenomena which occur in the development of occupational cancer from exposure to tar or X-rays, for example. The other forms of cancer for which no definite irritant can be identified at present are so similar in their mode of occurrence and the length of time necessary for their appearance that it is reasonable to accept the facts of experimental carcinogenesis as a model of the genesis of the others also. An attempt has been made to indicate directions in which difficulties still remain. The definition of these difficulties, and the material and methods by which they are being attacked, we owe to the modern development of experimental carcinogenesis.

Dr. J. W. Cook: In my contribution to this discussion I shall confine myself to the question of the experimental production of cancer by pure chemical compounds, and I propose to summarize and discuss the results obtained at the Cancer Hospital by my colleagues and myself under the direction of Professor Kennaway, with whom I wish these remarks to be associated. We agree entirely with Dr. Murray that the results provide no explanation of the mechanism of tumour production. This problem is a matter which is occupying our attention now. Furthermore, we regard the term "chronic irritation" as an entirely inadequate description of the effect of applying a carcinogenic substance to animal tissues. There are many examples of industrial skin diseases brought about by chemical irritation, but few of them lead to malignant tumours. There are many constituents of coal tar which are powerful irritants, for instance, many of the phenolic substances, yet these

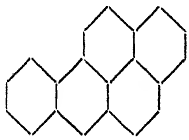
constituents are entirely without result in experimental carcinogenesis. Not one of the carcinogenic hydrocarbons has any apparent irritating effect on human skin, and there are many hydrocarbons with similar chemical and physical properties to the carcinogenic compounds which have no cancer producing activity whatever. If this activity, then, is to be attributed to chronic irritation, it is just as logical to attribute to the same cause the œstrogenic effect of certain synthetic compounds, as well as that of the natural hormone, œstrin.

Stress has been laid by Dr. Murray on the fact that other chemical and physical agents, such as X-rays and so on, can replace the constituents of coal tar. We have never lost sight of this, but these agencies do not seem to give any large proportion of tumours in the animals treated, and they provide very little scope for experimental investigation. Obviously, it is true that no view of the origin of malignant disease can be comprehensive if it fails to take into account the cancer-producing properties of all the known carcinogenic agents. For this reason experimental inquiries into the effect of various carcinogenic agents on tissue metabolism are of importance. The recent observations of Boyland that carcinogenic hydrocarbons can give rise to products which influence enzyme systems in a similar manner to arsenious acid which is undoubtedly responsible for some cases of cancer in man, are suggestive of the means by which correlation of the various carcinogenic agents may ultimately be attained.

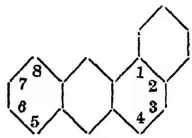
At this stage it will be profitable to direct attention to the chemical relationships existing between the synthetic cancer-producing compounds. The essential feature in these seems to be a condensed carbon-ring structure consisting of four or five aromatic rings arranged in the molecule. The few carcinogenic compounds which contain oxygen and nitrogen are so closely related to the allied carcinogenic hydrocarbons that for practical purposes it is sufficient to consider the group of carcinogenic hydrocarbons. These differ very greatly in potency as is shown by the fact that some of the compounds consistently give rise to tumours after a very much shorter interval than others. We are confident that these differences in incidence cannot be due entirely to solubility differences, although this last factor is clearly not without influence. The most powerful carcinogenic compound yet obtained is 1 : 2-benzpyrene, which we have recently isolated from coal tar, and also prepared synthetically.

Its formula (I) shows that 1 : 2-benzpyrene contains the ring-system of 1 : 2-benzanthracene (II), which has itself hardly any cancer-producing activity. A study of a very considerable number of hydrocarbons derived from 1 : 2-

benzanthracene has shown that carcinogenic activity, sometimes of a very high order, is conferred upon the molecule by attachment of alkyl groups at position 6, or of new rings to the 5 : 6-position. Thus, the 6-methyl and 6 : 7-dimethyl derivatives of 1 : 2-benzanthracene had slight activity; the



I.



II.

6-*iso*propyl derivative had very marked activity, which was also the case with 6 : 7-*cyclo*penteno-1 : 2-benzanthracene. Activity of a still higher degree was shown by 1 : 2 : 5 : 6-dibenzanthracene and 5 : 6-*cyclo*penteno-1 : 2-benzanthracene. By way of contrast one might point out that negative results were obtained with 15 simple derivatives of benzanthracene with substituents in other parts of the molecule, and negative results were also obtained with all excepting two of the twelve hydrocarbons which have so far been prepared, which have molecules consisting of five benzene rings condensed together. It seems clear that the property of cancer production is a constitutive property depending on a particular type of molecular arrangement.

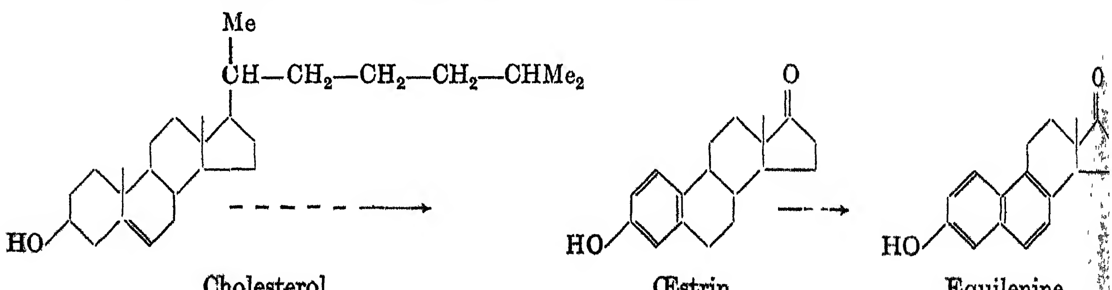
Application of these carcinogenic hydrocarbons, in benzene or other solvents, to the skin of mice, leads to the development of tumours, the malignant nature of which is shown by their microscopic structure, and by the fact that with some of the compounds a number of metastases have been obtained in the lungs and axillary glands. Graphical representation of the results of the experiments has shown in the case of the more active compounds the certainty with which the development of tumours may be expected, provided the animals live sufficiently long.

In addition to the epitheliomas arising from the application of these substances to the skin of mice, tumours of connective tissue have been obtained by injection of several of them. One of the mouse tumours produced in this way by 1 : 2 : 5 : 6-dibenzanthracene has now reached the sixty-seventh transplanted generation, and in rats the fortieth generation has been attained. Spindle-celled tumours, with metastases in the heart and other organs, have also been obtained by the injection of 1 : 2 : 5 : 6-dibenzanthracene into fowls.

A factor which is common to all the carcinogenic hydrocarbons is the presence of the phenanthrene ring-system, and it is of considerable interest that a number of naturally occurring compounds, some of them having considerable biological

activity, are now known to contain this particular ring-system. Among them are the sterols, the ovarian hormone, possibly also the male hormone, vitamin D, the cardiac poisons, and certain of the vegetable alkaloids. The elucidation of the correct molecular structure of the sterols and bile acids followed from the impetus given by the suggestions made by Rosenheim and King in May of last year, and it is now possible to visualize how these may be transformed by some abnormal metabolic process into carcinogenic agents. There is at present no evidence whatever that this actually occurs. I wish merely to indicate some of the possibilities.

Consider the various stages in the possible biological breakdown of cholesterol. In the first place, removal of part of the side chain by oxidation may give lithocholic acid, one of the bile acids. This in turn may be further oxidized to deoxycholic acid and cholic acid. Alternately, the process of side-chain degradation can proceed a stage further with the successive production of pregnandiol (only two carbon atoms remaining in the side chain) possibly the male hormone (side chain completely removed), then the female hormone, œstrin (one ring becoming aromatic), and finally the hormone, equilenine, which Girard has recently isolated from the urine of pregnant mares. In this last substance two rings have become aromatic:



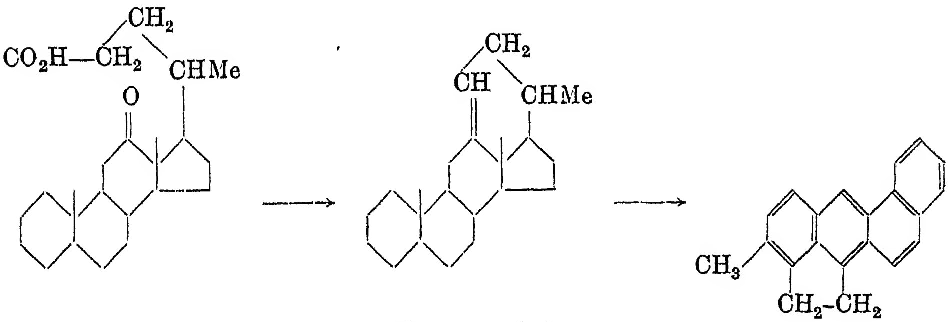
Cholesterol Œstrin Equilenine

This is as far as the chain of degradation reactions can be carried with substances which have actually been isolated from biological material. But one may imagine the process of aromatization of the rings to proceed a stage further, and the corresponding hydrocarbon with three aromatic rings may arise by reduction being effected at some stage. This hydrocarbon has been synthesized by Hewett and myself and is being tested for carcinogenic properties.

If this hydrocarbon, 1 : 2-*cyclo*pentenophenanthrene, should turn out to be carcinogenic, then the formation from œstrin of a carcinogenic compound would involve reduction of polar groups and aromatization of the ring-system. A correlation such as this between œstrogenic and carcinogenic activity

has been established for the 1 : 2 : 5 : 6-dibenzanthracene ring-system. Experiments carried out in collaboration with Professor Dodds have shown that the introduction of polar groups, coupled with partial reduction, can transform this carcinogenic hydrocarbon into a moderately potent œstrus-producing compound.

Even more suggestive is the possible conversion of a bile acid into a hydrocarbon of the 1 : 2-benzanthracene group by simple reactions (reduction, dehydration, dehydrogenation) which may readily occur in the animal body. Wieland has shown that 12-ketocholanic acid (from deoxycholic acid) may be dehydrated to dehydro*nor*cholene. Haslewood and I are investigating the dehydrogenation of this unsaturated hydrocarbon, which should lead to a benzanthracene hydrocarbon with substituents in positions 5 and 6 :



12-ketocholanic acid   Dehydro*nor*cholene

But we have seen that this type of structure is peculiarly favourable for the development of carcinogenic properties. If, therefore, this benzanthracene hydrocarbon proves carcinogenic, we shall regard it as a matter of first importance to learn much more than is known at present regarding the fate of the sterols and bile acids in the body. Speculation is not desirable except so far as it stimulates investigation, but it will be agreed that it is within the bounds of possibility that reactions such as those I have suggested may be facilitated by such agencies as ultra-violet light, radium, and X-rays.

Dr. W. Cramer : It is important in a discussion such as this to begin by trying to define what are the essential features of the phenomenon we try to explain. I can only in a short time be as summary as possible, and I should like to amplify once more what Dr. Murray has already said, that growth is a phenomenon which takes place even in the tissues of the adult body and is integrated to the needs of the organism, just as all the other activities of animal cells—

that is to say, it is subject to inhibitions and stimulations. There may be a temporary physiological hyperplasia, as in the uterine mucous membrane during œstrus or in the mammæ during pregnancy, which is due to a stimulus coming from without, and disappears when the stimulus ceases. In all these conditions, including the pathological hyperplasias, which are due to abnormal stimuli, the normal relationship to the supporting connective tissues is maintained so that the essential structure is not altered and the conditions either regress or become stationary when the stimulus from without disappears.

Malignancy is the negation of this integration. This condition progresses when the stimulus ceases. The malignant cell is no longer subject to the stimuli or inhibitions which control normal cells. The malignant cells do not observe the limitations placed upon the normal cells, and as a result of that we have invasion of the connective tissue and the formation of metastases.

As a result of this altered relationship we have the transplantability of malignant cells. If one takes a normal tissue and inoculates it from one animal to another of the same species a connective tissue reaction results which leads eventually to the disappearance and absorption of the inoculated transplant. Malignant cells elicit in the new host a connective tissue reaction which, by providing a stroma enables the malignant cells to establish themselves (in the new host) so as to form a new tumour. It is interesting to note that sometimes the connective tissue regains its dominance, and invades the tumour and the tumour disappears. That occurs in the very rare cases of spontaneous absorption. It is also the reaction by which one may obtain the disappearance of a tumour under the influence of radium. It is therefore probably due to a damage which the malignant cells have received so that temporarily they can no longer control the connective tissue.

In the short time at my disposal I can only summarize briefly the results obtained from the study of transplanted tumours in mammals. Dr. Murray has already pointed out that the malignant change is due to something within the cell, and that in mammals it is not possible to separate anything from the cell which can transmit the malignancy. Transplantation is not carcinogenesis. It is really a tissue culture of malignant cells *in vivo*. Their growth and behaviour are entirely divorced from the needs of the organism in which they grow.

There are many different types of malignancy, all of which breed true. That suggests that the cellular changes responsible for malignancy reside in the nucleus, the cell organ mainly responsible for inheritance. It also indicates that malignancy is not cell anarchy. The life and behaviour of the malignant

cell is not, as some writers have said, lawless, but it is governed by laws as strict as those which govern the life and behaviour of normal cells.

The phenomenon of carcinogenesis presents two problems, firstly, the nature of the cellular change which is responsible for the behaviour of the malignant cell, and, secondly, the manner in which this change can be brought about. While the nature of the cellular change is still unsolved, and is very much a matter of speculation and dispute, the problem of the manner in which this change can be induced is solved so far as the essential features are concerned. Cancer can be produced experimentally at will. If we except such diseases as tuberculosis, syphilis and vaccinia, which are caused by a specific organism, there are very few diseases which can be produced experimentally, and there are none in which the experimental production in animals simulates the natural disease so completely as it does in cancer. The same agents which are known to produce cancer in man have been found to produce experimentally cancer in animals, namely, gross parasites, physical agencies such as X-rays, radium, light, and chemical substances such as tar and lubricating oils. One and the same agency applied to one and the same tissue produces many different types of malignant growths. The period required to produce cancer when measured in actual time is very much shorter in the mouse (four to six months) than in man (ten to fifteen years), but when measured in biological time as a fraction of the normal span of life, it is almost identical. A long period of induction is necessary whatever agent is used, and may therefore be regarded as an essential feature of the process of carcinogenesis. The recognition of this fact is of great importance. It explains the age incidence of cancer, which is one of the most characteristic features of the disease, whether it occurs in man or in animals which must therefore be ascribed to the fact that the conditions which induce spontaneous cancer require a considerable fraction of the span of life of each species in order to produce a malignant transformation. Conversely, one may argue that since in man the great majority of patients develop cancer at or after the onset of middle age, the disease in man is due to conditions which operate over a long period.

The work of Professor Kennaway and his colleagues is an important advance in our knowledge so far as it has revealed the close chemical relationship which some of these carcinogenic agents have to the sterols, which are constituents of every normal cell. This suggests that normal cells can, if placed under abnormal conditions, themselves produce chemical substances of carcinogenic activity. It is possible that the conditions vaguely called chronic irritation which are known to produce malignancy, produce this effect by inducing the

cells on which they act to form carcinogenic substances as abnormal metabolites.

Another very important result of the experimental study of carcinogenesis has been the clear demonstration that the development of malignancy is determined not only by the action of carcinogenic agents, but also by the existence of a constitutional susceptibility of the individual organism, probably inherited. There is evidence that this factor of susceptibility plays an important part in the etiology of human cancer.

There is another but quite different way in which carcinogenesis can be induced experimentally. I refer to the transmission of tumours by cell-free extracts of certain tumours, the so-called filterable tumours of fowls. In the first place, this mode of carcinogenesis is immediate. Secondly, it is strictly specific in inducing only the same type of tumour as that from which the extract was obtained. Again, the agent present in these carcinogenic materials obtained from malignant cells is thermolabile and can give rise to antibodies. Further, this type of carcinogenesis has been observed only in birds and especially in fowls and has so far been found to be restricted there to tumours of mesoblastic origin. It has yet to be shown whether in fowls all tumours of mesoblastic origin can be transmitted in this way. Curiously enough, the appearance of spontaneous tumours in fowls does not seem to be effected in this way. As with mammals they appear in middle-aged or old individuals, whereas experimental transmission by filtrates is effected more easily in young birds than in old birds. They do not spread among healthy birds by contagion. In fact, clinically, these fowl tumours present all the features of malignant disease in mammals. The immunological work of Gye and Purdy and of Andrewes furnishes evidence, convincing to my mind, that the agent responsible for the transmission of these tumours contains an element which is not of fowl origin, in other words, a virus. The fact that this evidence also indicates the presence of a factor which is of fowl origin does not invalidate this conclusion. It suggests rather that the factor is a complex one, as Gye originally suggested.

This brings us directly to the consideration of the second question: the nature of the change in the cell which constitutes malignancy. In discussing the mammalian tumours we had come to the conclusion that this change must be a purely cellular one, not due to an extraneous agent, and that it probably affects the nucleus. Bauer has suggested that this change affects the genes so that the cancer cell may be said to represent a mutation of a normal somatic cell. This is an interesting guess which amounts, however, to little more than giving this nuclear change " a local habitation and a name." But it does not

enable us to understand the characteristic properties of malignant cells, such as their infiltrative growth or their altered metabolism.

We have arrived here at an apparent contradiction in the experimental evidence presented by mammalian tumours and by certain fowl tumours.

I feel that those who so confidently deny the possibility of a virus being responsible for malignancy take a somewhat narrow view, and over-estimate the extent of our knowledge concerning viruses. We know of the existence of viruses only so far as they are pathogenic, but just as there are non-pathogenic bacteria, there may be non-pathogenic viruses. Their presence cannot be recognized because they cannot be seen, and they do not produce a disease. But as the division between pathogenic and non-pathogenic bacteria is to a certain extent an artificial one—it is known that some bacteria can exist in the body without producing disease but can become pathogenic by inducing a specific lesion in the tissues—so it may be possible that a non-pathogenic intracellular virus may exist in normal cells without producing any change, but, may become pathogenic, perhaps by entering into the nucleus when the cell is placed under abnormal conditions. The pathogenicity of such viruses would depend on the condition of the cell and would not be inherent in the viruses themselves, so that it would not necessarily be demonstrable experimentally when the virus was brought in contact with the normal cell. So far as the disease would be elicited by a cellular change, such a conception would resolve the apparent contradiction, and the carcinogenesis by cell-free material from fowl tumours would then represent the final stage of the entire process of carcinogenesis.

There is yet a third conception which is based on experimentally established facts, and which seems to me to afford a better explanation of the disintegrated growth of malignant cells than the conceptions of a virus or a mutation. I refer to Warburg's work on the change of metabolism in malignant cells. The essential feature of this change is that it makes the malignant cell more independent of an available oxygen supply than the normal cells from which it is derived. Most normal cells cannot maintain their activities and live in the absence of oxygen as long as malignant cells, and the morphological structure of a normal organ provides for an adequate supply of oxygen for each individual cell under all conditions. The supply of oxygen is therefore probably one of the devices by which the activities and growth of normal cells are integrated. If now in a normal organ or tissue one cell changes its metabolism so as to become less dependent on the oxygen supply, it would afford an explanation of the disintegrated infiltrative growth which characterizes malignancy.

But whatever conception of the intracellular change we may adopt, we still have to explain why the normal cells of one and the same organ can give rise to such varied manifestations of malignancy, each of which breeds true. As I have pointed out, this problem presents itself most strikingly in the cell-free transmission of the filterable fowl tumours. The work of Gye is, so far as I know, the only attempt to deal with this difficulty by postulating the existence of a virus non-pathogenic in itself and modified in its action by some constituent derived from the malignant cell. The failure to separate completely the two factors is a failure to bring conclusive proof of the truth of this conception, but it does not disprove it, and nothing better has so far been put in its place. The recent immunological work of Gye and Purdy is indirect evidence of the complex nature of the agent capable of eliciting malignancy in fowls by tumour extracts, one part being of fowl origin, the other of non-fowl origin, that is, a virus.

It may appear at first sight that the alternative between a mutation, a change of metabolism, and a virus is rather a wide one, but the importance of this difference can be over-estimated. It would be possible, with sufficient speculation, to devise a theory which combines these three conceptions. The essential features of the disease have been fixed by the experimental study and are not affected whatever may be found to be the intimate cellular change. Cancer would remain a local and a non-contagious disease even if it were due to a virus. Therapeutically it would matter little. If it is a mutation we cannot mutate back at will, and if it is an intracellular virus we cannot kill a virus within the cell. An immune serum capable of neutralizing completely an active extract of the Rous sarcoma has no effect whatever on the cells of this tumour. The correction of an abnormal type of metabolism offers greater therapeutic possibilities, but so far attempts along this line have not been successful.

The solution of the ultimate "cause" of cancer might, therefore, not necessarily improve our methods of diagnosis and treatment. In fact, the differences encountered here have been allowed to obscure the great advance in our knowledge of the disease as a whole, which has a much more important bearing on the problem of dealing with the disease in man. Cancer begins as a local disease. It can then be cured. It is being cured in a high percentage of cases in those sites where it can be diagnosed most readily. We have learned that in the great majority of organs and tissues cancer is due to some extraneous factors setting up a condition of chronic irritation. If there are such extraneous factors it must be possible to avoid them on learning what they are. Cancer would then be a preventable disease.

Dr. C. H. Andrewes: It is becoming increasingly recognized that the filterable fowl tumours afford an important line of attack on the tumour problem as a whole. I find it difficult to believe that the fowl sarcomas are essentially of an entirely different nature from mammalian tumours, and I do not think it is possible to maintain the attitude that these filterable fowl tumours constitute a group of virus diseases in fowls quite distinct from fowl tumours as a whole. Whenever a fowl tumour has been studied over a reasonable period of time—by which I mean a year or two rather than a month or two—the tumour has sooner or later proved to be filterable. The filterable agents of these tumours can be neutralized by specific antibodies, and these antibodies may develop in the serum of tumour-bearing birds, or may be obtained by immunizing other animals. The study of these antibodies permits the serologist to take a closer part in cancer research than has been possible before.

Already serological studies have thrown light on a number of interesting points. First as to the nature of the agent in filtrates of fowl tumours; they behave in most respects like members of the virus group, but some people have been led to doubt whether they can be micro-organisms, because of the peculiar way in which the tumours arise. A filterable tumour will occur in a flock of birds spontaneously and sporadically, just as a mammalian tumour does, without any apparent relation to other cases of tumour in the flock of birds, and the experience is that if a number of tumours turn up over a period of time, each will be distinct histologically. That is not the sort of thing one expects if the agent is an infective micro-organism, and that is why a number of people have been led to suspect that the agent may not be a micro-organism of extrinsic origin, but something derived from the fowl cells. Serologists can point to two relevant facts in this connection. First of all, the body is not in the habit of producing an agent and at the same time producing an antibody to that agent, and this the tumour-bearing fowl must be doing if the agent has an intrinsic origin. In the second place, antibodies to these tumours, so far as they have been studied, behave exactly like antibodies to other viruses. They act apparently in the absence of complement, they have the same heat stability, they develop gradually in the course of an infection, they have a definite range of specificity, and they unite loosely with the antigen at first, and later more firmly. Therefore serological evidence does support the view that the agents are viruses.

The second point raised by Dr. Murray is that many people fight shy of a virus theory of cancer, because one would have to postulate the existence of an inordinate number of viruses, producing all sorts of histological types of

tumour throughout the animal kingdom. One naturally turns again to the fowl tumours, and asks whether the viruses producing difficult histological types of fowl tumour are all different from the point of view of the serologist. Unfortunately, the answer which the serologist gives to this question is not sufficiently definite; it is not "Yes" or "No." If one takes the sera from tumour-bearing birds, one finds that from birds with chronic tumours the serum will neutralize filtrates of quite a number of different histological types of tumour, so that the answer there seems to be, "Yes, they are all the same virus." But on the other hand the sera one obtains by immunizing pheasants are more specific than the fowl sera and two viruses which appear to be the same when tested with fowl sera are now found to be distinct. The bacteriologist is not very much surprised at finding that sort of thing, for he is familiar with bacteria which contain group and specific antigens and evoke preponderantly group or specific antibodies according to the animal immunized. But in any case the unsatisfying conclusion is reached that these different tumour viruses are neither identical serologically nor yet wholly distinct. There is something in them which is individual, and something which is shared by a number.

Gye and Purdy have obtained antibodies by immunizing goats with extracts of normal fowl tissues or normal fowl embryo. It is not surprising that they thus obtained anti-fowl antibodies, but it is surprising that these antibodies neutralized fowl tumour extracts. They only acted in the presence of complement, being heat-labile. At first sight their findings seem to be a strong argument in favour of the view that the filterable agents may be of fowl origin, but further work shows that the position is rather different. One fowl tumour (Fujinami's) can be propagated in fowls and also in ducks, and if one takes an extract of a tumour grown in the fowl one finds that it is neutralized by anti-fowl sera, and not by anti-duck sera; but on the other hand an extract of tumour grown in a duck, is neutralized by anti-duck sera, and not by anti-fowl sera. The neutralizability of the virus by anti-fowl serum is thus not an unvarying property. Gye and Purdy produced evidence that the association between the tissues and protein was closer than that of a mere mixture; they have also evidence that the association is with nuclear rather than with serum protein. Probably the evidence goes to show that the association between the tissues and protein is closer than has yet been demonstrated for any other virus. Gye has considered the question of whether this association may not in some way determine the cell specificity of these different tumours, and the hypothesis he has put forward is a very attractive

one, but one which is extremely difficult to test experimentally, and which remains at present unproven.

In conclusion, if a virus theory of cancer is worth considering at all seriously, one should also be prepared to accept the proposition that the viruses which cause tumours are probably not to be sharply differentiated from the viruses which cause infections. There are a number of conditions which the majority of cancer workers dismiss from consideration as being not true tumours which do seem to stand in an intermediate position between the tumours and infections. There are the infections, lymphosarcoma of dogs, the myxoma of rabbits, and also Shope's very interesting filterable fibroma of the rabbit, the agent of which apparently behaves at one time like a tumour producer and at another causes an inflammatory reaction which bears no resemblance to a tumour at all. These intermediate diseases are worth very serious study by everybody. When I referred to antibodies earlier in my remarks I spoke only of neutralizing antibodies. The reactions of complement fixation, precipitation and of agglutination of virus-bodies have not yet proved applicable to the study of the fowl tumours. Familiarity with current work on viruses should make us hopeful that these and other reactions will be applicable in the future, and if so serological study may help us to sort out these tumour-like conditions, and throw light on the really vital problem of the relation between the avian and the mammalian tumours.

Dr. P. R. Peacock: I think it has been fairly clear from what previous speakers have said that it is now recognized that the filterable avian tumours and their relationship to the mammalian tumours form a problem which must be solved before generalizations as to the cause of tumours can be regarded as more than very interesting speculations. The most obvious way of trying to settle these problems seems to be to take fowls and induce tumours in them by means which are recognized as producing tumours in mammals, and as this is a very obvious line of experiment, it is surprising that only about half a dozen fowl tumours have been so described, as far as I am aware, and only one of them has been carried beyond the second generation of transplantation. This tumour, produced in Murphy's laboratory at the Rockefeller Institute, was carried through eleven generations of transplantation, but was never shown to be filterable. Personally, I feel that inability to do something is not a proof that it cannot be done. Four years ago, I set about producing tumours with recognized carcinogenic samples of tar injected intramuscularly in fowls, and found no real difficulty at all in producing tumours, provided one could

keep the birds alive for from six months to a year. It is desirable that tumours produced in this way should be produced under circumstances which preclude the possible introduction of filterable agents, and I was fortunate in doing it in a field laboratory where no experiments on fowls had ever been carried out, so that no suspicion of that kind could be introduced with regard to my experiments.

In the very short time allowed me, the best thing I can do is to show by means of slides the general character of these experiments. The first slide illustrates experiments with tar injections. In the first series of 31 birds, 15 have died with tumours, 4 of which had metastasized. Three of these tumours have been successfully transplanted. In the next series, I injected dibenzanthracene suspended in lard; in 11 birds sarcomata resulted and 6 birds died with matastases, but only 2 of the tumours were propagated by transplantation. In the next series, I injected the same sample of dibenzanthracene suspended in yolk fat. A single injection was given to each of 12 birds, each injection containing $\frac{3}{4}$ mg. of the dibenzanthracene. One bird has developed a typical spindle-celled sarcoma. In the further series of 27 birds injected with tar, 5 have died with sarcomata, 3 of which had metastasized. The next table shows the sites of the metastases; it will be seen that lung, liver, and heart are the commonest sites. The next slide illustrates a typical transplantation experiment. A fowl was injected with tar, it developed a sarcoma and died with metastases. Material from it was injected into half a dozen birds. Three were grafted with intramuscular cell grafts, and two of the grafts grew. Two birds were injected with tumour material which had been thoroughly disintegrated by grinding up with sand, and the resulting pulp, diluted 1 : 50 in saline suspension was injected. A tumour grew in one bird. That bird was operated on, and other birds grafted from it. The grafts took fairly well in a certain number of birds and the tumour has now reached the sixth generation of transplantation. These saline suspensions are not, of course, guaranteed cell-free, but similar treatment of a mammalian tumour does not usually result in a positive finding. All attempts to transmit these tumours by means of filtrates have failed in my hands, but the tumour has reached only the sixth generation of transplantation.

My next slides give an idea of the type of tumour one gets. They show spindle-cell sarcoma following injection of a small amount of tar, also a secondary deposit in the liver. Another slide illustrates the primary tumour, in this case also a tar tumour, and the first, second, and third generation of graft tumours in small birds. Clinically and histologically, I believe, these were

indistinguishable from some of the filterable tumours. One of the birds in this series is interesting, because it was an old bird at the time it was inoculated. It was inoculated with saline suspension of a dibenzanthracene tumour. The grafts from this same tumour into young birds failed to take, but, injection of the ground-up tumour in an old bird took slowly and grew in five months to quite a large tumour in the wattle. That tumour was transplanted successfully in 1 out of 13 young birds. The next example is a tar tumour—a fairly typical spindle-celled sarcoma. The next is a Rous-like tumour, produced by dibenzanthracene.

I think that with regard to the mode of producing these tumours there can be no question that these growths in the fowl are exactly comparable with similar growths in mammals induced by similar means. They can be propagated, although they are difficult to propagate. If one can judge by analogy, it is fair to say, as far as experiments have gone, that the growths in the fowl are comparable with those in the mammal on the one hand, and with filterable fowl tumours on the other. Of course, the analogy does not prove that these tumours will be filterable, and that is where I must leave the matter at present.

Professor J. McIntosh: The work which I wish to lay before you to-day is the outcome of several years' experimentation on the virus theory of cancer. You have just heard that at present the chief basis for experimental work with regard to the virus theory is the so-called Rous sarcoma of fowls. At this hour of the discussion, it is not possible to go into the various arguments which have been raised for and against the neoplastic nature of the Rous sarcoma. We have heard to-day many different hypotheses, but, if I may say so, more definite facts are required—and I fully believe in the old adage that "the birth of a fact is the death of a theory." As Dr. Peacock has just said, it is essential to study the nature of tar-induced tumours in the fowl, and to compare them with the Rous sarcoma.

The experiments which I wish to bring to your notice have a direct bearing on the nature of these tumours and on the virus theory of cancer. In the experiments, a series of Plymouth Rock fowls, varying in age from four to six months old, were taken and injected with tar diluted in lard, so as to make a 2 or 4% dilution. Some four injections at 14 days' interval were made into the breast muscle on alternate sides, the average dose was 0·1 c.c. of the diluted tar, only in one series was pure tar used.

In the first experiment, six normal birds and four Rous-immune birds were used and kept in separate pens. Some of the birds died or were killed and

examined at periods of from 21 to 261 days after the first injection, others still survive, that is, for nearly a year now. In the second experiment, five birds, and in the third, six birds were used.

An examination of the protocols of these experiments, shows that tumours of one type or another were found in just over half (12 out of 20) of the birds treated with tar. In several instances macroscopic tumours were not found in the breast muscle, but it always showed evidence of cellular proliferation and infiltration. Some form of tissue proliferation was present at an early date after the tar injection, whilst in several instances, the remains of the tar in the muscle could be seen.

Microscopically, the tumours varied considerably in character, many being fibrosarcomatous in type, others leukosarcomatous with numerous indeterminate grades. Others again were predominantly fibro-endotheliomatous or even fibro-angeio-endotheliomatous. More curious was the tendency to obtain tumours of a pleomorphic character, or the association of fibrosarcomatous types with a leukosarcoma or a plain leukæmia. ("Leukæmia" is used here in the sense that the cells present are derived from the hæmopoetic system, and it was often difficult to say whether the cells were of erythroblastic or myeloblastic origin.)

In the Rous-immune birds no tumour of a Rous type was obtained but only those of a fibro-endotheliomatous or angeio-endotheliomatous type. Leukæmias were observed in both immune and non-immune birds.

At least three of the different types of tumours obtained by tar injection have been carried on through one or more passages; a fibrosarcoma, a fibro-endothelioma and a tumour of leukæmic elements. The fibrosarcomatous tumour, originally a leukæmia with only a fibrous reaction in the breast muscle, has been passed through six passages, and now seems to breed true, having completely lost the leukæmic element. For the first three passages the tumour was definitely of a mixed character. This tumour is a cellular fibrosarcoma of the Rous type and is readily filterable through a Berkefeld or Pasteur-Chamberland No. 2. It differs histologically from Rous sarcoma No. 1 in that it forms much firmer tumour with little or no myxomatous degeneration.

(The speaker here showed a number of slides dealing with the microscopic appearances of the tumours.)

Before discussing these results, I think it is necessary to say that, until we commenced these tarring experiments, we had not met with more than one spontaneous fowl tumours. This observation covers some seven years' work in which

it is safe to say over 5,000 fowls have been used. In the last few years, all the fowls have been obtained from the same breeder and are only kept in the laboratory for a few days before the experiment. This, together with the fact that several tumours were obtained by tar injections, negatives any suggestion that the tumours obtained were spontaneous in origin. Further, pleomorphism in certain fowl infections has been recorded by several workers. Thus, an analysis of the work of Ellermann, Furth, and others on leukæmias of birds shows that there is a good deal of pleomorphism in the type of disease produced by the inoculation of a leukæmic virus.

Thus, with a single strain of leucosis which Furth propagated in a large series of birds, he records that at one time or another it gave rise to a myeloid leucosis, a combination of myeloid and erythroleucosis, myeloma, sarcoma, erythro-leucosis, and lymphoid leucosis.

More recently, Oberling and Guerin found a similar pleomorphism during their propagation of a spontaneous strain of leukæmia. The virus, apparently affecting endothelial as well as fibrous structures, ultimately produced growth-like fibrous nodules ending finally in a sarcomatous tumour. This tumour, they say, is filterable and corresponds in type with the endothelioma of Murray and Begg.

The results of these experiments are capable of interpretation in a number of ways. Of these, three only will be considered.

It would seem that tar injections in some manner, by stimulating cell proliferation, allow the sarcoma and leukæmic viruses to get a hold on young cells of mesoblastic origin thereby initiating a continuous proliferation resulting in a leukæmia or sarcoma. The early local proliferative fibroblastic reaction with tumour-like appearance round the injected tar is well seen in the early lesions. It is, of course, necessary to presuppose that these viruses are widely distributed in the fowl world as, for instance, is herpes febrilis in the human subject.

Or, it may be that the proliferative and tumour-like effects are due to one single pleomorphic virus with an affinity for any tissue of mesoblastic origin. Thus, at one time, the result of its action may be a leukæmia, at another a fibro-sarcoma, at another an endothelioma, and so on—the action of a virus with an affinity for any mesoblastic tissue. The work of Andrewes and others on the close affinities of the various viruses of the different fowl tumours lends additional support to this conception.

The third is the acceptance of the specific factor of Gye; this specific factor, being derived from the tissues themselves and then linked with the common

virus of the fowl, determines the type of tissue proliferation—a leukæmia or a sarcomatous tumour.

From the above findings three main conclusions may be drawn :—

(1) That tumours can readily be induced in the fowl by intramuscular injections of tar, and that these tumours show a considerable pleomorphism in their histological appearance.

(2) That four tar-induced tumours have been transmitted in series by injections of tumour emulsion, and one by means of cell-free filtrates.

(3) That we have, as yet, much experimental ground to cover before we can formulate definite views as to the exact relationship between mammalian tumours and the virus growths of birds.

Dr. W. E. Gye : You have called upon me, Sir, but, in fact, I came to listen, and not to speak. There are, however, two observations which I should like to make. The first is with regard to leukæmias of the fowl to which Professor McIntosh has referred. I have worked with these for some time, and I can find no cross immunity relation between the Rous tumour and the virus of leukæmia. That is rather surprising to me.

One point on the subject of cancer that seems to be really rather important has not been touched upon yet by any speaker, and that is the work which has been carried out largely by Murphy and his collaborators on the inhibitory factor in the fowl tumours. The great variations in the rate of growth of tumours of a similar kind of structure furnish one of the outstanding puzzles of the cancer problem. If one considers the Rous sarcoma—the sarcoma grows at a great rate normally, but from time to time it enters into a benign stage, during which growth is very slow. How are we to explain this great variation in the rate of growth, together with the corresponding change in the clinical and pathological appearances ? Dr. Purdy and I have made the observation that the slow growing tumours contain something which exercises an inhibitory influence on filtrates of rapidly growing tumours. We could not devise a technique which would show this with satisfactory constancy, but Murphy apparently, from his papers, has obtained more constant results, and there appears to be no doubt that within the tumour cells an inhibitory substance is produced when the tumour grows slowly. The tumour cell responds to the presence of the virus apparently by producing something which will check and restrain the activity of the virus itself. That is of considerable importance in explaining small and great variations in rate of growth of a tumour.

One other point. If we are to make progress in the study of cancer we shall have to look inside the cell rather than confine our attention to the morphology of cells.

Professor A. E. BOYCOTT: There are one or two comments I should like to make. If I may go back to Dr. Murray's opening summary, I do not quite see that the three hypotheses he puts forward for the cause of cancer are alternative in the sense he uses them. They seem to me to be rather complementary to one another. I cannot quite see, for example, how Boveri's hypothesis of gene mutation can be the cause of cancer. I can see how it may be an expression of why a cancer cell is different from a normal cell. The more one thinks about that idea, the more attractive it is. Both Dr. Murray and Dr. Cramer laid stress on one aspect of the growth of tumours which is often forgotten. We have frequently said that tumours grow in an anarchical and lawless way, but we have overlooked the fact that they are only anarchical and lawless in the sense in which a criminal's conduct might be so described. The criminal has his own code, and sticks to it; it is different from that of normal people, but it is not strictly lawless. All our knowledge of transplantable tumours goes to show that a tumour maintains the same characteristics, so far as we know, for ever and ever. Those characteristics are handed on through successive generations of cells, and they are entirely different from the qualities of the normal tissue among which the tumour is growing. Surely, therefore, the cells of which the tumour is composed must differ genetically from the cells of the normal tissues. It is, at all events, a very convenient hypothesis, and I do not quite understand why Dr. Cramer asked, "What is the good of it?" It is the easiest way of understanding the point on which Dr. Cramer himself laid so much stress, that each tumour goes on being the same.

Taking that as the expression of why a cancer cell differs from a normal cell, the other two hypotheses again might be taken as not exclusive of one another in their explanations of the production of new growths. We have had some extraordinarily interesting information from the Cancer Hospital about these carcinogenic substances. It is known now that a great variety of physical and chemical agents have more or less carcinogenic power. This power is particularly marked in a group of substances represented originally by coal tar, and including shale oil, and the various things which are similar to those actually occurring in tar. It seems to me that the problem there is mainly this: Do these things act directly on normal cells and produce this mutation,

or do they all cause the production of some carcinogenic substance in the tissues? All these things cause inflammation, and inflammation can be brought about in many different ways. It is always manifested, however, in the same kind of way. From this we can deduce that it is not heat or cold or bacterial toxins or alkalis that cause the inflammation, but it is the products of the injury which they inflict on the cells which cause the change in the permeability of the capillary walls, the emigration of the leucocytes, and the other characteristics of the inflammatory process. Are these agents themselves carcinogenic because they produce carcinogenic substances, or do they act directly upon the cell? I gather from what Dr. Cook said that he is inclined to believe that they all produce a carcinogenic substance. I should have thought that the probabilities were against that, because of the great diversity of effects obtained: a great many tumours are very different from one another. But that is a question as to whether things are the same or different, and is a matter of opinion.

A third view as to the nature of these irritating substances regards them, I suppose, as affording a suitable nidus on which the supposed extraneous virus gets a lodging. The second and third hypotheses which Dr. Murray has brought forward are clearly two stages of the same hypothesis. I must confess again that I think the idea of an extraneous virus really has comparatively little to be said for it. There is not the slightest epidemiological evidence that any such virus exists. It seems to me very unlikely that a thing capable of independent life would stand the amount of knocking about that these agents will stand—precipitation, adsorption, re-suspension, and so forth. It is a remarkable live thing if it gets through to the end of that in a live state.

Directing my remarks particularly to what Dr. Andrewes had said, I think the antigenic argument can be very much overworked. If you make a compound of proteins with all sorts of chemicals they become antigenically different from what they were before. Surely a thing like a malignant tumour is very likely to be antigenic. The proteins of the lens of the eye, for example, are antigenic to the very same animal, from which it might be deduced that the proteins of the lens of the eye always contained a virus. That might be an illustration of Dr. Cramer's interesting speculation in which he talked about "new-pathogenic viruses in normal cells." I rather thought at the time that if one postulates a normal virus occurring in normal cells, one had better call it something other than a virus.

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*The Antiseptic and Trypanocidal Properties of some Anil and Styryl Derivatives of 4 Amino Quinaldine.**

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Biological Section.

In previous communications† antiseptic and trypanocidal properties of a number of amino derivatives of 2-anil and styryl quinoline have been dealt with. The majority of these contain amino groups, either primary, tertiary or acylated, in the para position of the benzene nucleus and the 6-position of the quinoline nucleus (or the 7-place in a few instances). In the present series the side chain in the quinoline nucleus is situated in the 4-position and consists of a primary, tertiary or acetylated amino group. Thus, several of these compounds are analogues of those already examined.

The methods of estimating antiseptic and trypanocidal action are the same as those previously employed† and the results are given in Table I. It has been shown that the most satisfactory indication of antiseptic potency is afforded by the "inhibitory concentration" of a substance, *i.e.*, the lowest concentration which prevents definite multiplication of the organisms, although all of the latter may not be killed; therefore only this value is given in the table.

General Considerations.

Antiseptic Properties.—With the 6-amino quinoline compounds it was found that those with a tertiary amino group in the para position of the benzene nucleus were more active than the corresponding primary amino compounds. Accordingly, all the substances in the present series are para dimethyl- or diethyl-amino derivatives. The group present in the 4-position in the quinoline nucleus is either a primary or tertiary amino, acetylamino, or β-diethylamino methochloride (or methiodide). Nos. 434, 428, 435, 431 and 440 correspond respectively with the 6-derivatives, Nos. 56, 62, 21, 24 and 37. The most

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† Browning, Cohen, Ellingworth and Gulbransen, 'Proc. Roy. Soc.,' B, vol. 100, p. 293 (1926); *ibid.*, vol. 105, p. 99 (1929).

No.	Substance.	Antiseptic action.				Precipitation.		Trypanocidal action.	
		Staphylococcus aureus.		*B. coli.*					
		P.	S.	P.	S.	P.	S.	Dose.	Result.
434	2 (*p*-dimethylamino anil) 4 amino quinoline methochloride	40	10	4	20	2	—	1 : 300 1 : 400	} (Cure).
428	2 (*p*-dimethyl amino anil) 4 acetyl amino quinoline metho-sulphate	100	40	10	20	4	—	1 : 1000	No action.
432	2 (*p*-dimethylamino anil) 4 diethyl amino quinoline metho-chloride	40	20	4	10	1	—	1 : 6000	No action.
438	2 (*p*-dimethylamino anil) 4 β diethyl amino methiodide ethylamino quinoline methiodide	10	4	<1	40	—	—	1 : 750	No action.
435	2 (*p*-dimethylamino styryl) 4 amino quinoline metho-chloride	100	100	<2	20	100	—	1 : 2000	No action.
431	2 (*p*-dimethylamino styryl) 4 acetylamino quinoline metho-chloride	40	20	4	10	2	—	1 : 1000	No action.
440	2 (*p*-dimethylamino styryl) 4 dimethylamino quinoline methochloride	400	400	20	100	4	—	1 : 30000	No action.
433	2 (*p*-dimethylamino styryl) 4 diethylamino quinoline metho-chloride	1000	400	20	40	4	—	1 : 10000	No action.
439	2 (*p*-dimethylamino styryl) 4 β diethylamino metho-chloride ethylamino quinoline methochloride	2	40	2	10	1	—	1 : 1000	No action.
441	2 (*p*-diethylamino styryl) 4 dimethylamino quinoline methochloride	1000	400	40	40	10	—	1 : 30000	No action.
442	2 (*p*-diethylamino styryl) 4 diethylamino quinoline metho-chloride	4000	400	40	20	10	—	1 : 15000	No action.

P = medium consisting of 0·7 % neutral peptone water. S = ox serum previously heated at 56° C.

Antiseptic action.—The numbers are the reciprocals ÷ 1000, of the concentrations which suffice to produce inhibition of growth in 48 hours at 37° C., so that the medium remains unclouded or shows at most very faint turbidity. In the former case subculture may yield no growth or, as in the latter case, may show very slight growth.

Precipitation.—The numbers are the reciprocals ÷ 1000, of the lowest concentrations which show precipitation in the media.

Trypanocidal action, as tested on *T. brucei* (a strain from Professor Mesnil, Institut Pasteur, Paris).—The doses are reckoned as 1 c.c. of the concentration shown per 20 gram body-weight of the mouse, injected subcutaneously 24 hours after inoculation, when scanty parasites were present in the blood. The highest dose shown for each substance is one which approximates to the largest amount borne by uninfected animals without producing obvious toxic effects, *e.g.*, loss of weight.

(Cure) indicates that with the doses shown cure was effected only in a proportion of the animals treated.

marked general feature of the 4-series is the weak action on *B. coli.* This is strikingly exemplified with No. 428 (2 (*p*-dimethylamino anil) 4 acetylamino quinoline methosulphate), when compared with the corresponding 6-analogue (No. 62), which has a very powerful antiseptic action on *B. coli.* In every instance the 6-analogue is the more potent. On the other hand, the tertiary amino quinoline derivatives of the styryl series have a strong antiseptic action on staphylococci (Nos. 440 433, 441, 442). But the β-diethylamino ethylamino derivative (439) is relatively weak.

Trypanocidal Effects.—No. 431 (2 (*p*-dimethylamino styryl) 4 acetylamino quinoline methochloride) has no action, whereas the 6-analogue (No. 24), although only slightly less toxic for the mammalian host, is a very effective trypanocidal agent and causes cure in a considerable range of dosage. This difference is a striking example of the relation between chemical structure and biological action in closely related compounds. The other styryl compounds are all practically inactive, but so also are the 6-analogues (Nos. 21, 37) in the doses tolerated. On the other hand, 2 (*p*-dimethylamino anil) 4 amino quinoline methochloride (No. 434) has led to cure, whereas the 6-analogue (No. 56), which is three times more toxic, has only a slight trypanocidal action. It is noteworthy, however, that the action of No. 434 is very erratic and the largest doses tolerated, which produce cure of the trypanosome infection in some animals, may be practically without effect in others similarly infected. The only other anil derivative which has ever proved curative is 2 (*p*-dimethylamino anil) 6 chloracetylamino quinoline methochloride (No. 71), although a number of them have a certain degree of action (Browning and others, 1926). In the case of the less toxic compounds, examination of the subcutaneous tissue at the site of injection did not support the view that the lack of trypanocidal action is due to non-absorption.

Chemical Section.

4 *hydroxyquinaldine* was prepared as described by Limpach (1932) with the exception that the ethyl β-phenylamino crotonate was heated with molten paraffin wax, and the latter extracted with boiling benzene or ligroin.

4 *chloroquinaldine* was obtained by heating the hydroxy compound with four times the weight of phosphorus oxychloride at 140°–150° (Conrad and Limapach, 1887).

4 *aminoquinaldine.*—4 chloroquinaldine (5 gm.) and saturated alcoholic ammonia (25 c.c.) were heated in a sealed tube at 190°–200° for 8 hours. The contents were diluted with water and evaporated to dryness, the residue

dissolved in HCl (5 c.c.) and water (30 c.c.) and the base precipitated with ammonia. The latter was filtered, washed and triturated with 100 c.c. of 10% caustic soda, and then filtered, washed and dried. It was crystallized from water. It forms clusters of colourless needles, melting point 167°–168°. The yield was about 70% of the theory.

4 *aminoquinaldine methiodide.*—Dimethyl sulphate (1·2 gm.) was added to a solution of 1·4 gm. of the amine in 5 c.c. of nitrobenzene, previously heated on the water bath and the whole was then heated for 1 hour. The methosulphate crystallized, and after filtering and washing with ether, was dissolved in water, and the methiodide precipitated by potassium iodide. This crystallized from water in clusters of colourless needles, which darkened on heating and decomposed at about 270°. The yield was 1·5 gm.

Found I = 42·0%. $C_{11}H_{13}N_2I$ = 42·3%.

4 *acetylaminoquinaldine.*—A mixture of the amine (5 gm.), acetic anhydride (10 c.c.) and fused sodium acetate (1 gm.) was heated on the steam bath for ¾ hour. It was poured into water (50 c.c.). Ammonia precipitated the oily acetyl compound which quickly solidified. It was filtered, washed with water and crystallized from water. It forms bunches of colourless needles, melting point 176–168°. The yield was 75% of the theory.

Found N = 14·1%. $C_{12}H_{12}ON_2$ requires N = 14·0%.

4 *acetylaminoquinaldine methiodide.*—This was prepared as described for the 4 amino compound, using 2 gm. of the acetyl compound and 1·3 gm. of dimethyl sulphate with 10–15 c.c. of nitrobenzene. A deep purple colour quickly developed, but this soon disappeared, and on further heating, the methosulphate separated. After ¾ hour, the mixture was cooled, filtered, washed with nitrobenzene and then with benzene or ether. It is a pale pink substance, very soluble in water. The yield was good.

The methiodide was prepared by precipitation with potassium iodide. It crystallized from dilute alcohol in long, colourless needles, which darkened on heating and decomposed at 274–275°.

Found I = 37·5%. $C_{13}H_{15}ON_2I$ requires I = 37·2%.

4 *dimethylaminoquinaldine methiodide.*—A mixture of 4 chloro quinaldine (5 gm.), 33% dimethylamine solution (10 c.c.) and alcohol (5 c.c. of 64 O.P.) was heated in a sealed tube at 200° overnight. The contents were diluted with water, and the volatile substances evaporated on the water bath. The residue was cooled and a solution of caustic soda (10 gm.) in water (50 c.c.) added. The oil was extracted with ether, dried over sodium sulphate, and the ether removed. The yellow oil was dried at 110° for ½ hour, and was used

directly for the preparation of the methiodide. Yield 4 gm. 5 gm. of the oil in 10 c.c. of nitrobenzene was heated on the water bath and then 3·5 gm. of dimethyl sulphate added and heated for 1 hour. It was cooled, diluted with water, and treated in the usual way. The methiodide crystallized from alcohol (64 O.P.) in clusters of pale yellow needles, which darkened on heating and decomposed at 290°–295°. The yield was 5 gm.

Found I = 38·9%. $C_{13}H_{17}N_2I$ requires I = 38·7%.

4 *diethylaminoquinaldine methiodide.*—A mixture of 4 chloroquinaldine (5 gm.), absolute alcohol (5 c.c.) and diethylamine (8 c.c.) was heated at 200° overnight in a sealed tube. The alcohol was evaporated and the residue dissolved in hot dilute HCl. It was made alkaline with ammonia, extracted with ether and dried over sodium sulphate. The ether and diethylamine were distilled off. The diethylamino quinaldine remained as a thick yellow oil, which was dried at 110° for ½ hour. Yield 5 gm. The methiodide was prepared exactly as described for the dimethyl compound, using 1·9 gm. of the oil and 1·1 gm. of dimethyl sulphate. The yield was 1·6 gm. The methiodide crystallized from water in pale yellow rods, which melted at 157°–159°. It is easily soluble in hot water, and alcohol.

Found I = 35·8%. $C_{15}H_{21}N_2I$ requires I = 35·7%.

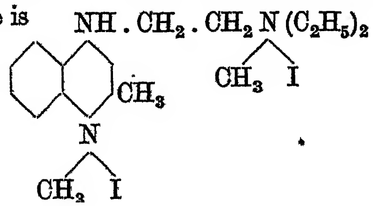
4 β-*diethylaminoethylamino quinaldine.*—A mixture of 4-chloroquinaldine (5 gm.), absolute alcohol (15 c.c.) and $NH_2 . CH_2 . CH_2N(C_2H_5)_2$ (4 gm.) was heated in a sealed tube at 190°–200° for 7–8 hours. The contents were evaporated and the thick gummy residue taken up in HCl (5 c.c.) and water (20 c.c.). It was filtered and ammonia added. The thick creamy oil was extracted with ether, the solution dried over sodium sulphate and the ether distilled. The residual brown oil was dried at 110° for ½ hour. Yield 5 gm.

The *dimethiodide* was prepared exactly as described for 4 acetyl amino quinaldine methiodide, using 6·9 gm. of the oil and 3·4 gm. of dimethyl sulphate. It crystallized from dilute alcohol in clusters of needles, which darkened at about 260° and decomposed at 279°.

Found I = 46·5%. $C_{17}H_{26}N_3I$ requires I = 31·8%.

$C_{18}H_{29}N_3I_2$ requires I = 46·95%.

Hence the substance is



The *anils* in this series were prepared by the usual method, only a longer time of heating was necessary, generally about 6 hours. The yields were rather poor.

The *styryls* were all prepared by melting together equal weights of the methiodide and the aldehyde, at 140° in the presence of some piperidine. The yields were 50–60%.

The methochlorides of the styryls were quite soluble in water giving reddish-brown solutions, while the anils gave deep red or reddish-purple colours.

2 (p-*dimethylamino anil*) 4 *amino quinoline methiodide* (No. 434)—crystallized from alcohol in irregular brown prisms with a green reflex, and was only slightly soluble in alcohol.

Found I = 29·5%. $C_{19}H_{21}N_4I$ requires I = 29·4%.

2 (p-*dimethylamino anil*) 4 *acetyl amino quinoline methiodide* (No. 428).—This was prepared from the corresponding methosulphate. It crystallized from absolute alcohol in clusters of purple needles with a green reflex.

Found I = 26·3%. $C_{21}H_{23}ON_4I$ requires I = 26·8%.

2 (p-*dimethylamino anil*) 4 *diethyl amino quinoline methiodide* (No. 432)—crystallized from absolute alcohol in long chocolate coloured needles.

Found I = 25·5%. $C_{23}H_{29}N_4I$ requires I = 26·0%.

2 (p-*dimethylamino anil*) 4-β-*diethyl amino methiodide ethylamino quinoline methiodide* (No. 438)—crystallized from alcohol in brownish-purple needles.

Found I = 37·5%. $C_{26}H_{37}N_5I_2$ requires I = 37·7%.

2 (p-*dimethylamino styryl*) 4 *amino quinoline methiodide* (No. 435)—crystallizes in brick red prisms from a large volume of 64 O.P. alcohol.

Found I = 29·4%. $C_{20}H_{22}N_3I$ requires I = 29·5%.

2 (p-*dimethylamino styryl*) 4 *acetylamino quinoline methiodide* (No. 431)—crystallized in brownish-purple needles from 64 O.P. alcohol.

Found I = 26·5%. $C_{22}H_{24}ON_3I$ requires I = 26·85%.

2 (p-*dimethylamino styryl*) 4 *dimethylamino quinoline methiodide* (No. 440)—crystallized from 64 O.P. in clusters of red needles.

Found I = 27·8%. $C_{22}H_{26}N_3I$ requires I = 27·7%.

2 (p-*dimethylamino styryl*) 4 *diethylamino quinoline methiodide* (No. 433)—crystallized from 64 O.P. in long red needles.

Found I = 25·7%. $C_{24}H_{30}N_3I$ requires I = 26·1%.

2 (p-*dimethylamino styryl*) 4 β-*diethylamino methiodide ethylamino quinoline methiodide* (No. 439)—crystallized from a large volume of boiling 64 O.P. in masses of fine red needles.

Found I = 37·25%. $C_{27}H_{38}N_4I_2$ requires I = 37·8%.

2 (p-*diethylamino styryl*) 4 *dimethylamino quinoline methiodide* (No. 441)—crystallized in clusters of red needles from 64 O.P.

Found I = 26·1%. $C_{24}H_{30}N_3I$ requires I = 26·1%.

2 (p-*diethylamino styryl*) 4 *diethylamino quinoline methiodide* (No. 442).—The red melt obtained by fusing the two components in the presence of piperidine was very soluble in absolute alcohol, and on adding excess of ether, the styryl was precipitated as a green sticky mass. This was washed several times with ether and then dissolved in a small amount of absolute alcohol, and allowed to evaporate in the air. When completely dry, the solid was crystallized from a very small volume of boiling 64 O.P. It crystallized in clusters of red needles with a bronze green reflex.

Found I = 24·9%. $C_{26}H_{34}N_3I$ requires I = 24·7%.

The various methiodides or methosulphates were converted into methochlorides by treatment with silver chloride in suspension in alcohol as described in former communications.

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*The Antiseptic and Trypanocidal Action of some Benzoylamino Quinoline Anil and Styryl Compounds.**

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Biological Section.

The antiseptic and trypanocidal properties of certain amino derivatives of 2-styryl quinoline have already been dealt with.† Hitherto the substituent groups in the quinoline nucleus have been chiefly in the 6-position (occasionally the 4- or 7-position) and have consisted of primary or tertiary amino, acyl-amino or carboxylamino groups. In the present series the 6-position is occupied by a primary, tertiary or acetylated para amino benzoylamino group. The methods of estimating antiseptic and trypanocidal action correspond with those previously used.† The results are given in Table I. For reasons already stated (Ashley and others, p. 293), the antiseptic potency is represented by the inhibitory concentration of the substances.

Antiseptic Properties.

The anils in which the 6-amino group of the quinoline nucleus is arylated (Nos. 73, 422, 421) are all very powerfully antiseptic toward both staphylococcus and *B. coli*. In this respect they resemble the 2 *p*-dimethylamino anils in which the 6-position in the quinoline nucleus is occupied by an acyl-amino group (Nos. 59–69). Several of the styryl compounds also are fairly active antiseptics both for staphylococcus and *B. coli*, especially Nos. 426, 430. On comparing their effects with those of the non-benzoylated analogues

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† Browning, Cohen, Ellingworth and Gulbransen, 'Proc. Roy. Soc.,' B, vol. **100**, p. 293 (1926); *ibid.*, vol. **105**, p. 99 (1929); Browning, Cohen, Ashley and Gulbransen, *ibid.*, vol. **110**, p. 249 (1932); Browning, Cohen, Cooper and Gulbransen, *ibid.*, vol. **110**, p. 372 (1932); Ashley, Browning, Cohen and Gulbransen, p. 293.

bearing the same terminal groups in the side-chains, it appears that there is no close parallel between structure and action in the two series—*cf.* Nos. 245, 87, 427, 21; 426, 24; 437, 90. Several of the benzoylamino compounds even in very high dilutions are precipitated in protein solutions (peptone water, serum); this is especially so with No. 430 and, to a less extent, with Nos. 421, 437, 422 and 245.

Toxicity and Trypanocidal Action.

The substances most readily precipitated by peptone water or serum *in vitro,* also form after injection into the subcutaneous tissue coloured deposits which persist for long periods. There is a tendency for necrosis of the skin to result; but necrosis seldom occurred with No. 430. The low toxicity of these compounds is probably due to the minute amounts which circulate in the body at a given time. In the case of No. 430, some months after the injection the material forms a mass of the colour of the original substance, with a consistency resembling that of oil paint, most of which can be easily scraped out of the connective tissue. It is readily suspended in water and appears as minute, elongated, coloured granules measuring under 1 up to several μ. No. 245 causes a greater tissue reaction and the stained mass becomes enmeshed in connective tissue from which it cannot be so readily removed. With both substances the local staining has been apparent over a year after the injection and in the case of No. 430 the development of sarcoma has been observed locally; but with No. 245 this has not occurred in 15 animals observed for 11 to 14 months after the injection. The anil derivatives (Nos. 421, 422 and also No. 73*) have some trypanocidal action, but do not effect cure. Of the styryl compounds those with a dimethylamino group in the benzene nucleus (Nos. 426, 427) are without trypanocidal action; the others are actively trypanocidal. No. 245 is fairly uniform in its effect, and the ratio of the dose which is tolerated to that which may produce cure is greater than 240 : 1. No. 437 does not act in so wide a range of dosage. The effect of No. 430 is highly irregular; while a dose of 0·00025 gram may cure the infection in a mouse of 20 gram weight, on the other hand, 0·0066 gram may be entirely without effect. These substances, apparently on account of their persisting as a "depot" in the subcutaneous tissues, exert a prolonged prophylatic action. In order to give an indication of the sensitiveness to therapeutic agents of the strain of *T. brucei* used, it should be added that with similar animals treated at a corresponding stage of

* The maximum tolerated dose of No. 73 is 1 : 250.

Table I.

No.	Substance.	Antiseptic action.				Precipitation.		Maximum tolerated dose.	Trypanocidal action.	
		Staphylococcus aureus.		*B. coli.*						
		P.	S.	P.	S.	P.	S.		Dose.	Result.
422	2 (*p*-dimethylamino anil) 6 (*p*-amino benzoylamino) quinoline methochloride	>1000	200	>1000	400	40	4	>1 : 150	1 : 150–1 : 600	Slight—marked.
421	2 (*p*-dimethylamino anil) 6 (*p*-acetylamino benzoylamino) quinoline methochloride	>200	>400	>1000	100	100	40	>1 : 150	1 : 150	No action.
245	2 (*p*-acetylamino styryl) 6 (*p*-amino benzoylamino) quinoline methoacetate	100	10	1	10	20	2	>1 : 100	1 : 100–1 : 1000 1 : 2000–1 : 24000 1 : 25000 1 : 50000	Cure. (Cure). Slight—marked. No action.
427	2 (*p*-dimethylamino styryl) 6 (*p*-amino benzoylamino) quinoline methochloride	40	10	10	20	1	1	1 : 1000	1 : 1500	No action.
426	2 (*p*-dimethylamino styryl) 6 (*p*-acetylamino benzoylamino) quinoline methochloride	40	100	100	40	4	<1	1 : 1000	1 : 1200	No action.

430	2 (*p*-amino styryl) 6 (*p*-acetylamino benzoylamino) quinoline methoacetate	100	200	20	100	1000	100	>1 : 50	1 : 150–1 : 4000 1 : 5000–1 : 6000	No action—marked—(cure). No action—marked.
437	2 (*p*-acetylamino styryl) 6 (*p*-dimethyl-amino benzoylamino) quinoline methoacetate	<3	20	<3	<3	200	20	>1 : 100	1 : 500–1 : 2000 1 : 3000–1 : 7500	Cure. Marked.

P = medium consisting of 0·7% neutral peptone water. S = ox serum previously heated at 56° C.

Antiseptic action.—The numbers are the reciprocals ÷ 1000, of the concentrations which suffice to produce inhibition of growth in 48 hours at 37° C., so that the medium remains unclouded or shows at most very faint turbidity. In the former case subculture may yield no growth or, as in the latter case, may show very scanty growth.

Precipitation.—The numbers are the reciprocals ÷ 1000, of the lowest concentrations with which precipitation occurs in the media.

Maximum tolerated dose.—The dose shown is approximately the most concentrated solution of which a mouse weighing 20 grams will tolerate 1 c.c. injected subcutaneously without showing obvious toxic effects, *e.g.*, loss of weight.

Trypanocidal action as tested on *T. brucei* (a strain from Professor Mesnil, Institut Pasteur, Paris)—a subcutaneous injection of 1 c.c. per 20 gram mouse being given 24 hours after inoculation, when scanty parasites were present in the blood.

Lesser degrees of trypanocidal action are designated as follows:—

Slight = disappearance of parasites from the blood for several days to a week.

Marked = absence of parasites from the blood for 10 days or longer.

(Cure) indicates that with the doses shown cure was effected only in a proportion of the animals treated.

the infection with *tryparsamide* (sodium salt of N-phenylglycineamide-*p*-arsonic acid) the following results were obtained :—

Dose per 20-gram mouse.	No. of animals treated.	Result.
0·01 gram	1	1 cured
0·0066 "	2	1 cured 1 slight action
0·005 "	4	2 marked action 2 slight action
0·004 "	4	4 slight action
0·0033 "	4	3 slight action 1 no action
0·0025 "	9	3 slight action 6 no action

Four rabbits which had been inoculated 2 to 3 weeks previously and showed evidence of infection, as well as having parasites in their blood, received each a single subcutaneous dose of 0·02–0·025 gram per kilogram of body weight of No. 245 as a 0·7–0·8% solution in water; no necrosis of skin resulted. All were cured.

Chemical Section.

6 p-*nitro benzoylamino quinaldine* was prepared by dissolving 10 grams of *p*-aminoquinaldine in glacial acetic acid on the water bath and adding a solution of 12 gm. of *p*-nitro benzoyl chloride in glacial acetic acid. The mixture was heated for half an hour and after standing some time to cool the precipitated hydrochloride was filtered, suspended in water and made alkaline with ammonia, filtered, washed with water and dried. 20 gm. of crude *p*-nitro benzoyl amino quinaldine were thus obtained as a white powder, which is pure enough for conversion to the amino derivative, melting point 266°–267° C. It crystallizes from nitrobenzene in pale yellow crystals. Found N 13·7%. $C_{17}H_{13}O_3N_3$ requires N 13·7%.

6 p-*amino benzoyl amino quinaldine* was obtained by reducing the 20 gm. of *p*-nitro compound in suspension in 90% acetic acid with 15 gm. of iron filings on the water bath for half an hour. The amino compound dissolved as it was formed and the precipitated iron products were removed by filtration, washed with dilute acetic acid and the diluted filtrates made alkaline with ammonia. The precipitated mixture of quinaldine compound and ferrous hydroxide was filtered, washed and extracted with boiling alcohol, filtered through a hot fluted filter, reheated to boiling and diluted with water until cloudy. On

cooling colourless needles or plates crystallized. Melting point 197°–199° C. Found N 15·2%. $C_{17}H_{15}ON_3$ requires N 15·2%. Yield 11 gm.

6 p-*amino benzoyl amino quinaldine methochloride* (Method I).—6 gm. of the base were dissolved in nitrobenzene on the water bath and 2·4 c.c. of dimethyl sulphate (redistilled below 100° C. *in vacuo*) added. After 10 minutes heating the solution was cooled and filtered, washed with nitrobenzene and benzene and dried on the water bath. The 6 gm. of methosulphate thus obtained were dissolved in the minimum quantity of hot water, filtered and precipitated with an equal volume of saturated salt solution. After cooling, filtering and washing with water, the product was dried. It can be crystallized from a large volume of water and forms small yellow needles, but is pure enough to use for condensation without crystallization.

6-p-*amino benzoyl amino quinaldine methochloride* (Method II).—11 gm. of amino quinaldine methochloride (prepared by the hydrolysis of the acetyl derivative) were dissolved in glacial acetic acid on the water bath and 8 c.c. of pyridine added. 10 gm. of *p*-nitrobenzoyl chloride in glacial acetic were then added and the mixture raised to the boiling point and refluxed for 3 hours. After allowing it to stand overnight, the crystallized product was filtered, suspended in 100 c.c. of hot water and basified with ammonia. The resulting *p*-nitro benzoyl amino quinaldine methoacetate was crystallized from 50% alcohol, forming long plates. Yield 12 gm.

This product was dissolved in 40 c.c. of 90% acetic acid and 9 gm. iron filings slowly added, the mixture being heated on the water bath between each addition and finally heated for half an hour and filtered hot. The residue was washed with a further 20 c.c. of 90% acetic acid and the solution allowed to crystallize. The crystallized portion was dissolved in hot water, made alkaline with ammonia and precipitated by salt as a bright yellow precipitate (yield 2·9 gm.). The iron oxide residues were further washed with water, the wash liquors and the acetic acid mother liquors, made alkaline with ammonia, boiled with charcoal, filtered hot and precipitated with salt (yield 7·1 gm. crude). The whole material was recrystallized from water in long yellow needles—total yield 7·0 gm. Found Cl 10·4%, $C_{18}H_{18}ON_3Cl$ requires 10·8%.

6 p-*dimethyl amino benzoyl amino quinaldine methochloride* (Method II).—6-(*p*-amino benzoyl amino) quinaldine methochloride (3·3 gm.) were suspended in a solution of 1·1 gm. of anhydrous sodium carbonate in 50 c.c. of water and 2·0 c.c. of freshly distilled dimethyl sulphate added, heated for 5 minutes on the water bath and then boiled for 10 minutes. Saturated salt solution was added and when cold the greenish yellow precipitate filtered off. The

product was suspended in absolute alcohol and water added drop by drop while boiling, till dissolved. The solution crystallized on cooling in bunches of pale greenish yellow needles (1·4 gm.). Found, Cl 10·3%, $C_{20}H_{22}ON_3Cl$ requires 10·0%.

6 p-*acetyl amino benzoyl amino quinaldine* was obtained by suspending the finely powdered amino compound in 5-6 times its weight of acetic anhydride and a little fused sodium acetate. After heating for 1 hour on the water bath, the product was filtered, washed with water and dilute ammonia and dried. The white powder was used directly for the preparation of the methochloride. It crystallized from alcohol in stout needles. Melting point above 300° C. Found N 13·3%, $C_{19}H_{17}O_2N_3$ requires N 13·2%.

6 p-*acetyl amino benzoyl amino quinaldine methochloride.*—4 gm. of the base were dissolved in the minimum quantity of nitrobenzene at 150° C. (about 250 c.c.) and 1·6 c.c. of freshly distilled dimethyl sulphate added. The temperture was maintained at 150° C. for a quarter of an hour and the mixture then allowed to cool slowly. The product was then treated as for the *p*-amino methochloride preparation.

6 p-*dimethyl amino benzoyl amino quinaldine methiodide* (Method I).—8·4 gm. of the amino benzoylamino quinaldine were suspended in a solution of 3·6 gm. anhydrous sodium carbonate in 50 c.c. of water. 10 c.c. of dimethyl sulphate were added and the mixture warmed gently. A vigorous reaction started and a clear solution resulted. The solution was boiled for 10 minutes and then evaporated to a small bulk. A solution of 15 gm. of potassium iodide was next added and the yellow precipitate of methiodide filtered. It was crystallized from about 1 litre of water. It forms yellow microscopic crystals. Found I 27·9%, $C_{20}H_{22}ON_3I$ requires I 28·4%.

Condensations with p-*dimethyl amino,* p-*amino and* p-*acetyl amino benzaldehydes.*

The following conditions had to be adhered to in all these condensations since the adoption of the usual methods (ethyl alcohol and water to dissolve the quinaldinium salt) gave dark coloured, black or even tarry products.

3 gm. of the dry powdered methochloride were suspended in about 100 c.c. of methyl alcohol, a slight excess of aldehyde and 1 c.c. of piperidine added. The mixture was then gently refluxed for 6 hours and filtered hot. The resulting chloride or iodide was too insoluble in some cases and was converted to the methoacetate by refluxing in methyl alcohol with a slight excess of

silver acetate for 1 hour. The methoacetate was then dissolved out with hot methyl alcohol and distilled off at a low temperature till crystallization took place.

The following products were prepared :—

2 (p-*acetylamino styryl*) 6 (p-*amino benzoyl amino*) *quinoline methoacetate* (245) —orange red crystals, giving an orange solution in water. Found N 11·2%, $C_{29}H_{28}O_4H_4$ requires 11·3%.

2 (p-*dimethyl amino styryl*) 6 (p-*amino benzoyl amino quinoline methochloride* (427)—dark green needles, giving a violet solution in water. Found Cl 7·4%, $C_{27}H_{27}ON_4Cl$ requires Cl 7·7%.

2 (p-*dimethyl amino styryl*) 6 (p-*acetyl aminobenzoyl amino*) *quinoline methochloride* (426)—dark brick-red crystals with slight green reflex, giving violet red solution in water. Found Cl 6·6%, $C_{29}H_{29}O_2N_4Cl$ requires 7·1%.

2 (p-*amino styryl*) 6 (p-*acetyl amino benzoyl amino*) *quinoline methoacetate* (430)—brick-red crystals with slight green reflex, giving red solution in water. Found N 10·9%, $C_{29}H_{28}O_4N_4$ requires 11·3%.

2 (p-*acetyl amino styryl*) 6 (p-*dimethyl amino benzoyl amino*) *quinoline methochloride*—orange-yellow crystals, giving a yellow solution in water. The methochloride prepared from the dimethylamino benzoyl amino quinaldine methochloride made by method II was purer than that from a product prepared by method I. Found Cl 7·1%, $C_{29}H_{29}O_2N_4Cl$ requires 7·1%.

The corresponding *methoacetate* (437) was obtained by refluxing the methochloride in alcoholic solution with silver acetate, filtering and evaporating till crystallization took place. Orange crystals, giving a yellow-orange solution in water.

Condensation with p-*nitroso-dimethylaniline.*

Anil condensations took place satisfactorily with the usual conditions (ethyl alcohol and water to dissolve the quinaldinium salt).

2 (p-*dimethylamino anil*) 6 (p-*acetylamino benzoylamino*) *quinoline methochloride* (421)—long needles with copper reflex, changing to a green reflex on drying on water bath, and dissolving in water to a deep blue solution. Found Cl 7·2%, $C_{28}H_{28}O_2N_5Cl$ requires 7·1%.

2 (p-*dimethylamino anil*) 6 (p-*amino benzoylamino*) *quinoline methochloride* (422)—crystals with a green-gold reflex, giving a blue solution in water. The yield was poor.

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A Spectrographic Investigation of the Metallic Content of the Liver in Childhood.

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This paper describes the results of an investigation into the metallic content of the human liver during intra-uterine life and childhood, carried out by the spectrographic method of analysis described by Ramage (1929). A total of 111 children's livers, varying in age from birth to 12 years, and 14 fœtal livers, were examined. Of these, 100 specimens were obtained from the post-mortem room of the Hospital for Sick Children, Great Ormond Street, London, and we are greatly indebted to the medical staff of the Hospital for allowing us to make use of this material. We are also indebted to the Maternity Department of the Royal Hospital, Wolverhampton, and to the Birmingham and Midland Hospital for Women, for fœtal specimens.

Every liver was weighed at the post-mortem after removal of the gall-bladder, and a small piece was taken for analysis and weighed. It was then dried to constant weight in a steam oven, and the spectrum obtained by burning in ashless filter paper a weighed amount (usually 0·025 gm. or 0·05 gm.) in an oxy-coal gas flame with a silica burner, before the slit of a quartz spectrograph. A quantitative estimation was made by comparing the intensities of the lines so obtained with those derived from burning in a similar manner varying amounts of a standard solution. The standard solution had the same composition as that employed in a previous analysis of human tissues (Sheldon and Ramage, 1931). The comparisons were made (1) directly on the plates using the most suitable lines, the weaker, or the more refrangible when a choice was possible, and (2) on prints on a gaslight printing paper using three or even four prints made with different exposures. The relative intensities of the reversed lines and bands on the prints could be determined in this way with very considerable accuracy. It has been proved by experiments that the quantity of filter paper used, 0·11 to 0·12 gm., is adequate to give spectra of the standards comparable quantitatively with the spectra of the tissues. The actual degree of accuracy attained in estimating the percentages of the metals in the specimens varied somewhat with different elements; in the worst estimations it would be within

10% and in the best well within 5% of the larger quantities recorded. The results will be described with especial reference to the metals Fe, Cu, Mn, Rb, Ca, and Ag. Although with the data available it was an easy matter to calculate the percentage of the various metals in the fresh tissue, no reference is made to them in this paper, all the percentages results referring to the dry tissue. The livers employed were not from normal bodies but diseased, death being caused by a great variety of pathological states, many of which had already led to a considerable loss of water from the tissues, thus introducing a source of variation which it was thought desirable to exclude by dealing only with tissues dried to a constant weight. The extent to which the figures are applicable to healthy children is not known.

Data regarding the mineral content of human tissues from healthy bodies is urgently required; tissues from diseased bodies, although not apparently affected by the disease, can only be viewed with suspicion. The analyses of a large number of such tissues, however, would possibly furnish figures from which mean results might be calculated and age or other curves drawn, and the analyses of the rare cases of healthy tissues obtainable could be used to check the accuracy of those curves. The results detailed below must be studied in the light of these facts; they vary within limits according to factors more or less obscure, dependent on the reactions of the body to the disease.

This particular method of spectrographic examination does not yield lines due to zinc that are easy of detection and no opinion is offered with regard to this element. The results are set out in detail in Table VII.

Iron.—This metal was present in every case though in No. 41—a case of cirrhosis of the liver—the amount present was too small to enable a comparison to be made with the standard solutions.

The effect of age on the amount of iron present in the liver is illustrated by fig. 1, the average figures on which it is based being in Table I.

(*a*) The fœtal store of iron is brought about by an increase in the percentage of iron during the first 6 months, and by the growth in size of the liver during the last 3 months, the percentage remaining almost stationary during this period. The variations in individual iron content are greatest at birth, and become much less thereafter.

(*b*) The rise during the first 2 months after birth is probably due to the storage of iron set free by post-natal blood destruction, as suggested by Gladstone (1932), while the sharp drop during the remainder of the first year is caused by the demands made on the iron reserve by the formation of hæmoglobin and the growth of the body.

Table I.

Age period :	Foetal. 0–24 (weeks).	Foetal. Full-term.	Months. 0–1	1–2	2–3	3–6	7–8	9–10	11–12	13–24	25–60	61–108	109–144
% Fe	0·21	0·25	0·30	0·374	0·280	0·190	0·144	0·119	0·053	0·048	0·062	0·088	0·088
Total Fe (mg.)	15·2	73·3	80·5	74·1	71·7	68·9	68·3	64·8	31·1	33·6	76·2	141·2	173·0
Number of examples	6	6	10	15	9	9	7	10	7	14	8	10	7

Table II.

Age period :	Foetal. 0–24 (weeks).	Foetal. Full-term.	Months. 0–1	1–2	2–3	3–6	7–8	9–10	11–12	13–24	25–60	61–108	109–144
% Cu	0·016	0·03	0·026	0·026	0·017	0·014	0·011	0·009	0·0078	0·0068	0·0069	0·0058	0·006
Total Cu (mg.)	1·33	7·26	6·85	5·66	4·33	5·46	5·7	5·48	4·3	4·65	7·4	9·04	12·9
Number of examples	7	8	11	14	9	9	8	10	7	14	8	10	7

(*c*) The continuation of the drop into the second year is obscure, as by this time the food should be sufficiently varied to allow of an increase in the iron intake. It may be an error in sampling owing to the comparatively small numbers, or it may be that the morbidity rates being higher for children whose tissues are deficient in iron (Mackay, 1931), the figures represent individuals who started life with an initially low iron reserve, and chanced to die during this particular age period. It is probable that the figures of this age period cannot be transferred to healthy children, and this is especially true of the total figures, since in this group there were several instances of marasmus, with notably sub-normal weights.

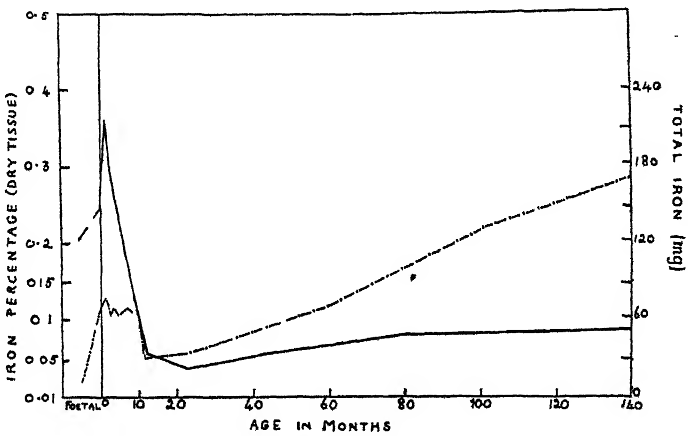

Fig. 1. —— iron per cent.; — · — · — total iron.

(*d*) The importance of this fœtal store of iron is shown by the fact that it is not until about the fifth year that the average child regains as much iron in the liver as it possessed at birth.

Copper.—This metal was estimated quantitatively by comparisons made with the 3274·0 and 3247·5 lines. It was present in measurable amounts in all the specimens examined.

Variation with Age.—This is illustrated by fig. 2, the average figures on which it is based being given in Table II.

(*a*) Copper differs from iron in that its percentage nearly doubles during the last 3 months of fœtal life. The infant is therefore even more dependent on the later months of pregnancy for its store of copper than it is for iron.

(*b*) Although there is no immediate post-natal rise, copper resembles iron in that the sharp decline in values does not begin until about 2 months after

birth, and that it is not until about the fifth year that the average child regains as much copper in the liver as it possessed at birth.

Manganese.—This element was measured by the triplet (4030·8, 4033·1 and 4034·5). It was present in measurable amounts in all the specimens except those derived from fœtal livers during the first 6 months of development.

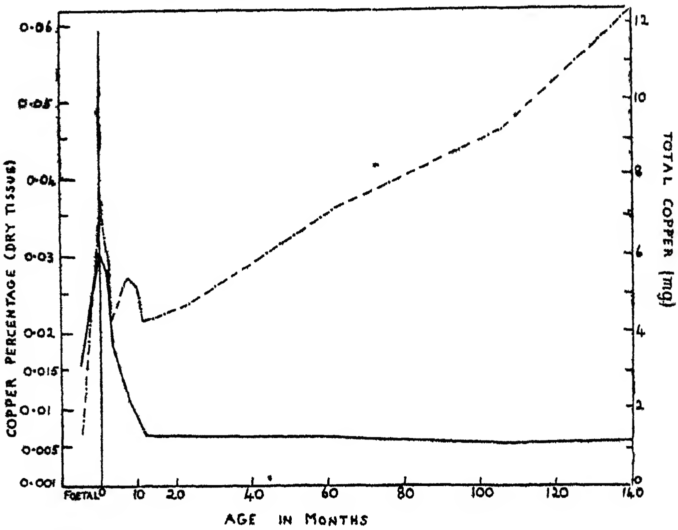

FIG. 2. —— copper per cent.; — · — · — total copper.

The variation of manganese with age is quite different from that of iron and copper, as may be seen from fig. 3. The average figures on which it is based are given in Table III.

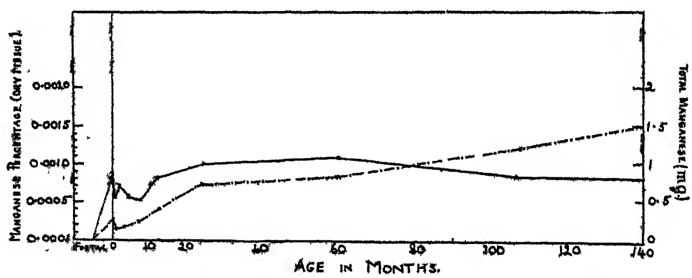

FIG. 3. —— manganese per cent.; — · — · — total manganese.

There is a marked rise in the percentage of manganese during the last 3 months of fœtal life. The drop following birth is attributable to the low manganese content of milk, since there is an immediate rise after 9 months, when a milk diet is supplemented by other food. This rise reaches the birth

Table III.

Age period :	Fœtal.		Months.								
	0–24 (weeks).	Full-term.	0–1	1–2	3–4	5–8	9–12	13–24	25–60	61–108	109–144
% Mn	Trace	0·0009	0·00053	0·00070	0·00057	0·00052	0·0008	0·0010	0·0011	0·00085	0·0008
Total Mn (mg.) ..	?	0·28	0·13	0·14	0·19	0·25	0·3	0·71	0·78	1·27	1·5
Number of examples	7	8	10	17	13	12	17	13	10	10	7

level, and the curve differs sharply from those of iron and copper, in which the birth level is never regained. The human infant is not provided with an initial store of manganese, and with the exception of the nursing period the values for manganese are remarkably constant throughout the whole of the age-period examined. In the case of manganese, the amount present at birth is exceeded before the end of the first year.

Rubidium.—This was estimated by its 4201·8 and 4215·6 lines. The lines were present in 123 of the 125 specimens but were not seen in No. 2, a child of

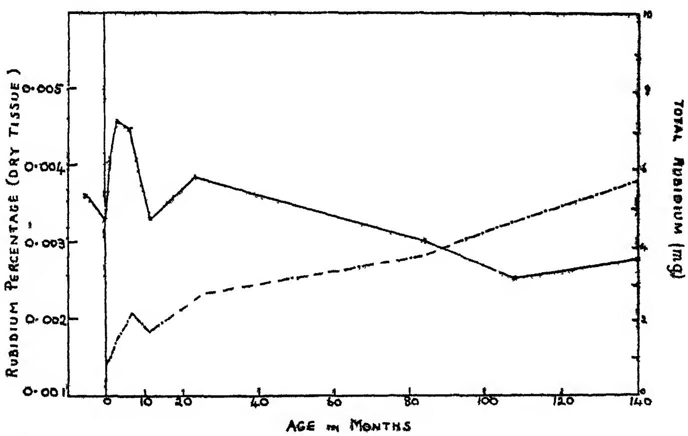

Fig. 4. —— rubidium per cent.; —·—·— total rubidium

9 months dying from hydrocephalus, and in F.13, a fœtus of 24 weeks' development.

The variation of rubidium with age is shown in fig. 4. The average figures on which it is based are given in Table IV.

The variations in the average percentage of this element are of slight extent, lying between 27 parts and 46 parts per million. This metal is not stored during fœtal life, and offers a complete contrast with the other metals described, in that the highest readings occur during the nursing period, which suggests

Table IV.

Age period :	Fœtal.		Months.								
	0–24 (weeks).	Full-term.	0–2	2–4	4–8	8–12	13–24	25–84	85–108	109–144
% Rb	0·0037	0·0033	0·0042	0·0046	0·0045	0·0033	0·0038	0·0031	0·0027	0·0029
Total Rb (mg.)	?	0·94	1	1·5	2·1	1·76	2·8	3·7	4·48	5·7
Number of examples	5	6	25	14	11	16	11	9	7	7

that rubidium may have some special significance at this time. The presence of rubidium in human milk was shown by Wright and Papish (1932) and was confirmed by ourselves in earlier investigations (Ramage, 1929; Sheldon and Ramage, 1931).

Silver.—The 3382·9 and 3280·7 lines of silver were present in 35 of the 111 children's livers, and in 5 of the 14 fœtal livers. In only one instance was the line sufficiently strong to allow of an estimation of the quantity present (No. 30—0·0025%). As silver appears to be present in only 32% of the specimens, it would seem, at first sight, doubtful whether it has any physiological significance. If, however, it is only an accidental contaminant, it is a remarkably constant one, since in our previous examination of 200 human tissues (Sheldon and Ramage, 1931) it was present in 47% of the livers examined, as well as in many other tissues. If it is of physiological importance, these findings must be taken to mean that the amount in which it is normally present is too small to allow of its identification by the spectrographic method employed, the instances in which the lines appear representing the presence of unusually large amounts.

Metallic Content of Fœtal Gall-bladder and Meconium.

In all the fœtal specimens, the gall-bladder was dried after washing out the bile, and examined in the same way as the liver. Table V expresses the average results.

Except for manganese, the liver has a higher percentage metallic content than the gall-bladder, whereas with manganese the position is reversed, the full-term gall-bladder containing 1½ times as much manganese as the liver. The examination of three specimens of meconium throws some light on the reason for this difference. All the specimens of meconium contained only minimal traces of iron indicating that there was a practically complete retention by the fœtus of all the iron provided by the mother. The presence of considerable

amounts of iron in the fœtal gall-bladder therefore implies that if iron is excreted by the bile during fœtal life, there is almost complete re-absorption.

In view of the close similarity between copper and iron, it is to be expected that meconium would also be poor in copper, but the reverse is actually the case. The copper lines are strong, and indicate the presence of 0·02% of copper in the dry meconium. This shows that during fœtal life copper is excreted into the bowel and the large amount found in the gall-bladder indicates that this excretion is by way of the bile.

The spectrum of meconium, however, is chiefly remarkable for the strength of the manganese triplet, which indicates a percentage of 0·008—a figure in excess of any so far found by us in human tissues, either in this investigation or in our previous one with 200 specimens (Sheldon and Ramage, 1931). One

Table V.

Age period:	0–24 weeks.		Full-term.	
	Gall-bladder.	Liver.	Gall-bladder.	Liver.
Iron %	0·045	0·21	0·12	0·25
Copper %	0·008	0·016	0·014	0·03
Manganese %	Traces	Traces	0·0014	0·0009
Rubidium %	0·0022	0·0037	0·0028	0·0033

cannot but associate this finding with the fact that manganese is unique in having a greater concentration in the gall-bladder than in the liver; there is evidently an active excretion of manganese during fœtal life by way of the bile. It would appear from these observations, and from the general smooth nature of the manganese curve in fig. 3 that the organism is concerned to maintain its content of manganese within comparatively narrow limits, and anything in excess of these, is, even in fœtal life, excreted. The spectrum of meconium also shows strong lines due to calcium and magnesium.

Correlation between Different Metals.

It was evident from an inspection of the spectra that there were many specimens in which iron and calcium varied in quantity together, and a chart drawn with the percentages of calcium plotted against those of iron fully confirmed the observation. A series of similar charts was then drawn and significant relations were found to exist between iron and calcium, iron and copper, and consequently between calcium and copper, and to a less extent

between potassium and rubidium. Apart from these pairs, the other metals appeared to vary independently.

Iron-Calcium.—The general nature of the correlation between iron and calcium is shown in fig. 5, which is based on the analyses of the 100 specimens collected at Great Ormond Street. The mean curve was obtained by plotting the average percentage of calcium found for each percentage of iron. Only 49 specimens contained more than 0·10 per cent. of iron, and these, being so scattered, were grouped in three sets in order to get better average results; a larger number of results in this region is desirable. The dotted lines represent a percentage of calcium varying by plus or minus 0·04% from the corresponding figure in the mean curve. The 19 cases lying outside these limits are represented by individual points. Eighty-one of the 100 cases fell within

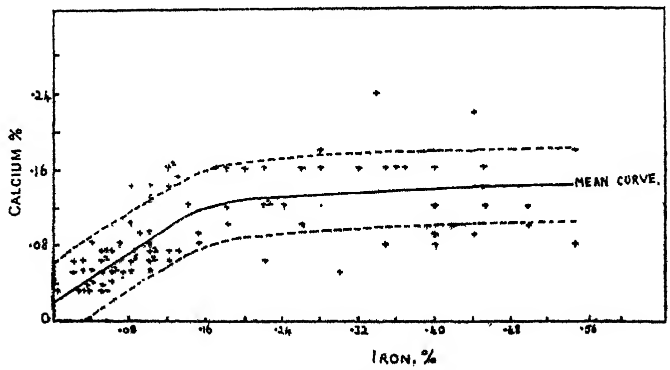

FIG. 5.—Iron-calcium

these limits. With due regard to the caution expressed on p. 309 it is evident that there is a definite relation existing between iron and calcium, the two regardless of age-difference, rising together.

Taking those cases in which the percentage of calcium differs by more than 0·04% from the mean curve, there are 10 specimens (Nos. 2, 8, 15, 16, 45, 49, 77, 78, 85, 96) in which calcium is distinctly high compared with iron, and 9 specimens (Nos. 5, 11, 52, 62, 63, 64, 65, 71, 84) in which it is low.

All the cases in the second group were under 8 months old. It is to be observed that there is only one case where the iron is as high as 0·30% (the average figure found during the first month after birth) where the calcium is below 0·08%. This suggests that the relation may prove to be one-sided, in that without the presence in the tissues of an adequate amount of calcium the foetus or child is unable to store iron. It should be pointed out that Sherman (1932) states that Von Wendt 30 years ago found that a liberal intake of calcium

appeared to conserve iron in metabolism, and refers to unpublished studies by Quinn and himself as showing that the ability of the growing organism to gain iron at an optimal rate depended not merely on the iron content of the diet, but also on its calcium content. Our findings are in agreement with these statements.

Iron-Copper.—In view of the fact that both of these metals are stored in the liver during fœtal life, it is to be expected that they would show some degree of correlation. The degree of correlation is given in fig. 6 in which all the individual points are marked and the approximate mean curve is drawn as in the iron-calcium chart, from the average percentages of copper plotted against

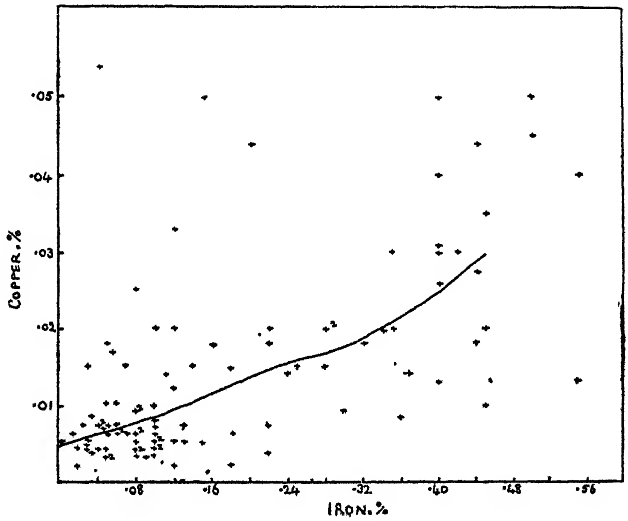

FIG. 6.—Iron-copper.

the percentages of iron. In general, the percentage of one metal tends to increase with the other: 0·02% of iron corresponds to 0·0055% of copper and 0·45% of iron with 0·03% of copper. The points representing the individual specimens are much more scattered round the mean line, especially in the higher percentages of iron, than in the iron-calcium chart. Both copper and iron fall rapidly in the first year of life, but it is evident that they do not vary in the same ratio in every case. Very extreme cases are seen in two instances of congenital heart disease, Nos. 90 and 68 with very high copper and low iron, and in a premature 8 months child (No. 54) with 0·15% of iron and 0·05% of copper. There are two cases of the reverse condition with a high iron and low copper content (No. 22—pyloric stenosis and No. 1—cystic disease of the kidney).

Calcium-Copper.—The relationship between these metals is shown in fig. 7 and it will be seen that there are more extreme differences, cases of low calcium and high copper and of high calcium and low copper, than in the pairs of metals in the preceding figures. The approximate mean curve rises from 0·03% calcium with 0·007% copper to 0·16% calcium with 0·0188% copper. The chart has a closer resemblance to the iron-calcium chart in that while there are numerous instances where a high calcium is found in association with a low copper, the specimens having a high percentage of copper show a distinct

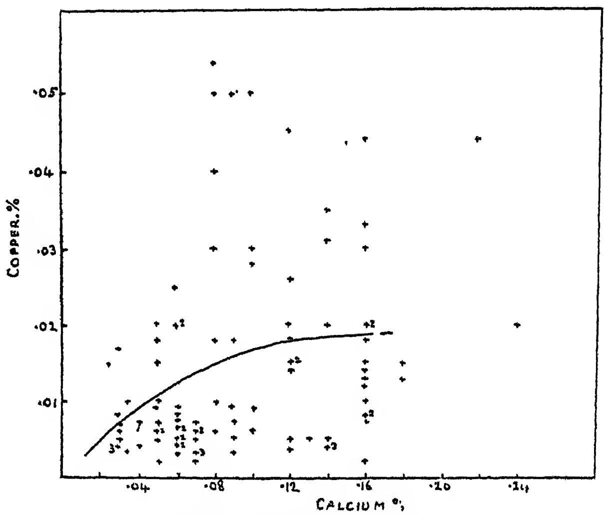

Fig. 7.—Copper-calcium.

tendency to have a high calcium. Thus a copper value of 0·03% (the normal average at birth) does not occur below a calcium value of 0·08%, and it would almost appear as if this were a limiting figure since with both copper and iron the average birth figures only occur in the presence of at least this amount.

These three charts suggest that copper, iron and calcium are in some way interdependent and there are indications that the relationship as between calcium and the other two is one-sided, *i.e.*, the tissues are only able to take up copper and iron in adequate amounts in the presence of a requisite percentage of calcium. It is a striking fact that there are 20 cases in the 100 in which the percentages of iron and calcium are about equal and the percentage of copper in every case is one-tenth of them. The aggregates of the three sets of figures are: iron, 1·435; calcium, 1·365; and copper, 0·1405. The ages vary from 2 months to 8½ years, so that age cannot be an important factor.

Potassium-Rubidium.—These two metals showed a similar, though less

marked relationship, which is presumably attributable to their close chemical similarities.

Variations in Metallic Content of the Liver with Disease.

Owing to the fact that (*a*) both the various diseases and the metallic content of the liver are subject to different modes of variation in respect of age, and (*b*) that there is no true normal level for comparison, great difficulties are encountered in attempting a correlation between the various diseases found in Table VII and the metallic content of the liver. In two cases, however, the results are sufficiently definite to merit discussion.

Pyloric Stenosis.—Seven cases (Nos. 5, 22, 26, 39, 52, 53, 61). There is a consistently high percentage of iron, while copper though usually high is more variable. A remarkable feature is that with the exception of No. 5 all the cases show a high content of rubidium. This is shown in Table VI.

Table VI.

Number of case	5	22	26	39	52	53	61
Rb %	0·0016	0·006	0·008	0·005	0·007	0·008	0·004
Age	7/52	6/52	6/52	2/12	2/12	3/12	7/52

These figures must be regarded as significant, since in the 111 diseased livers, only seven had a rubidium content in excess of 0·006%, of which three are included in this disease group. Only four cases reached 0·008%, which was the maximum figure found in the investigation, and two of these are to be found above. No. 5 is the only instance in which the rubidium content is below the normal for the age-group. It seems therefore that pyloric stenosis tends to be associated with a high content of rubidium in the liver, which in certain cases may reach extreme limits. The significance of this observation is quite obscure. It is not due to wasting, since in 14 cases of marasmus in the same age-groups the figures were, with one exception, either normal or subnormal. The average figure for these marasmus cases was 0·0037% as against 0·0053% for the cases of pyloric stenosis.

Pink Disease.—Two cases (16, 80). The values for all the metals were within normal limits.

Congenital Cirrhosis of the Liver.—One case (41). This was a case of congenital hypertrophic cirrhosis of the liver of "hobnail" type in a child dying at the age of 8 months. The liver was large, weighing 320 gm. The results are of great interest, since the disease is rare, and so far as we have been able

Table VII.

No.	Age.	Disease	Copper.		Manganese.		Iron.		Rubidium.		Calcium.		Silver.
			% Cu dry.	Total Cu in mg.	% Mn dry.	Total Mn in mg.	% Fe dry.	Total Fe in mg.	% Rb dry.	Total Rb in mg.	% Ca dry.	Total Ca in mg.	
1	2 wks.	Polycystic kidneys	0·01	1·2	0·0007	0·08	0·45	57·6	0·0024	0·27	0·16	20·5	—
2	9 mths.	Hydrocephalus	0·005	2·49	0·0001	0·05	0·10	53·8	None	None	0·13	71	—
3	7 mths.	Miliary Tb.	0·005	3·7	0·0004	0·59	0·03	23	0·0016	1·18	0·06	46·1	—
4	11 wks.	Subcutaneous abscess	0·03	4·2	0·0004	0·12	0·4	60·9	0·0016	0·24	0·16	24·3	—
5	7 wks.	Pyloric stenosis	0·03	7·65	0·0005	0·2	0·42	102·5	0·0016	0·4	0·10	26·1	—
6	2 yrs. 4 mths.	Tb. meningitis	0·017	12·1	0·0009	0·6	0·055	39	0·004	2·79	0·03	21·7	—
7	2 wks.	Intracranial hæmorrhage. Marasmus	0·02	7·3	0·0008	0·28	0·12	44·3	0·0016	0·56	0·06	21·5	—
8	6 wks.	Pyonephrosis	0·03	11·2	0·0008	0·3	0·12	27·6	0·004	1·5	0·16	60·7	Yes.
9	11 mths.	Diarrhœa	0·004	1·9	0·0005	0·26	0·04	17·5	0·0016	0·76	0·04	17·5	—
10	8 mths.	Miliary Tb.	0·007	2·1	0·0009	0·28	0·22	69	0·0024	0·75	0·16	51	—
11	1 mth.	Congenital hydrocephalus	0·02	4·4	0·0008	0·18	0·22	48·5	0·006	1·3	0·06	13·1	Yes.
12	6 wks.	D. and V.	0·02	3·9	0·0008	0·15	0·28	53·7	0·006	1·08	0·16	31·5	—
13	4 wks.	Congenital syphilis	0·02	2·7	0·0007	0·10	0·28	40	0·004	0·6	0·14	19·9	Yes.
14	11 yrs.	Rheumatic carditis. Cerebellar abscess	0·004	8·6	0·0006	1·2	0·08	104·7	0·003	6	0·03	66·6	—
15	7 yrs. 5 mths.	Rheumatic pancarditis	0·004	7·39	0·0005	0·92	0·1	179·5	0·0014	2·5	0·14	253·4	—
16	1 yr. 1 mth.	Pink disease	0·012	6·8	0·0008	0·44	0·1	57·4	0·002	1·15	0·16	88·8	—
17	1 yr. 7 mths.	Tb. meningitis	0·0035	2·2	0·0012	0·74	0·08	48·9	0·0035	2	0·06	36	—
18	10 mths.	Acute nephritis	0·025	10·2	0·0008	0·31	0·08	31	0·004	1·5	0·06	22·28	—
19	6 yrs. 4 mths.	Rheumatic carditis	0·003	4·5	0·0007	1·00	0·09	134·5	0·002	3·02	0·09	126·1	—
20	1 yr. 6 mths.	Tb. meningitis	0·008	6·9	0·0008	0·69	0·035	27·2	0·005	4·05	0·03	23·1	—
21	4 mths.	Congenital syphilis	0·009	3·2	0·001	0·35	0·08	28	0·004	1·4	0·06	21·03	—
22	6 wks.	Pyloric stenosis	0·013	2·3	0·0008	0·14	+0·55	99·8	0·006	1·04	0·18	28·98	—
23	8 yrs. 6 mths.	Tb. meningitis	0·008	9·7	0·0008	0·97	0·09	106·8	0·005	5·8	0·06	72·87	—
24	2 mths. (3 wks. premature)	Prematurity	0·015	3·4	0·0002	0·045	0·14	32	0·006	1·3	0·12	25·3	—
25	8 yrs. 5 mths.	Parenchymatous nephritis (chronic)	0·004	8·9	0·0008	1·79	0·1	232	0·0016	3·58	0·06	137·3	—

26	6 wks. (3 wks. premature)	Pyloric stenosis	0·035	4·7	0·0008	0·11	+0·45	63·6	0·008	1·1	0·14	19·2	Merest trace.
27	9 mths.	Mastoiditis. Peritonitis. (pneumococcal)	0·006	6·6	0·0008	0·88	0·08	88	0·0025	2·8	0·06	62·9	Yes.
28	2 wks.	Pemphigus neonatorum (not syphilitic)	0·028	11·6	0·0008	0·31	+0·25	101·8	0·006	2·48	0·10	40·7	Yes.
29	3 mths	Gastro-enteritis	0·026	5·72	0·0008	0·17	0·4	88·8	0·0045	0·98	0·12	26·6	Yes
30	2 mths.	Gastro-enteritis	0·031	4·5	0·0008	0·11	0·4	60·72	0·006	0·89	0·14	20·7	0·0025%
31	9 mths.	D. and V.	0·015	6·4	0·0008	0·34	0·18	74	0·004	1·7	0·12	50·3	Merest trace.
32	5 mths.	Congenital heart	0·007	3	0·001	0·37	0·04	16·9	0·007	3	0·05	18·8	—
33	2 yrs.	? Cœliac	0·0035	1·9	0·0016	0·9	0·035	19·6	0·003	1·7	0·035	19·6	—
34	7 mths.	Pyelonephritis	0·018	8	0·0008	0·3	0·22	101·5	0·0035	1·5	0·12	49·7	Merest trace.
35	4 mths.	D. and V. Mastoiditis	0·014	6·9	0·0009	0·4	0·24	120	0·0075	3·5	0·12	58·8	Merest trace.
36	5 mths.	D. and V.	0·006	1·9	0·0008	0·25	0·18	60	0·003	1	0·10	31·2	—
37	1 yr. 1 mth.	Empyema	0·007	4·4	0·002	1·3	0·06	38·3	0·005	2·9	0·04	26	Yes.
38	9 wks.	Marasmus	0·014	3·2	0·001	0·2	0·37	86·3	0·005	1·1	0·16	36·3	—
39	2 mths.	Pyloric stenosis	0·013	2·5	0·001	0·2	0·4	80·9	0·005	1	0·16	31·7	Yes.
40	3 yrs. 6 mths.	Acute appendix	0·007	9·3	0·0012	1·5	0·04	51·8	0·004	5·1	0·03	40·4	Yes.
41	8 mths.	Congenital cirrhosis of liver	0·005	3·4	0·0001	0·06	Trace	Trace	0·002	1·3	0·03	20·5	—
42	1 yr. 9 mths.	Rickets. Broncho-pneumonia	0·006	2·6	0·001	0·4	0·015	6·2	0·003	1·2	0·03	13·02	—
43	1 yr. 8 mths.	Tb. meningitis	0·007	7·3	0·001	0·9	0·025	26	0·002	2·3	0·03	31·2	—
44	1 yr.	Pyelitis. D. and V. Otitis media	0·015	7·7	0·0016	0·7	0·03	15·5	0·003	1·5	0·025	12·9	—
45	3 wks.	Congenital anæmia. Jaundice	0·02	8·5	0·0002	0·08	0·34	143·1	0·003	1·3	0·24	102	Yes.
46	4 mths.	Gastro-enteritis	0·008	2·8	0·0005	0·18	0·36	131·9	0·006	2·1	0·16	56·7	Yes.
47	3 yrs.	Broncho-pneumonia	0·006	5·2	0·001	0·8	0·07	58	0·002	1·6	0·08	70·5	—
48	3 yrs.	Influenzal meningitis	0·005	6·4	0·001	1·09	0·08	104	0·008	10·9	0·06	76·7	—
49	9 yrs.	Rheumatic carditis	0·005	8	0·0016	2·5	0·12	193·3	0·002	3·4	0·14	226·9	—
50	2 wks.	Gangrene neonatorum	0·02	6	0·0001	0·03	0·35	106·9	0·003	0·9	0·16	50·32	—
51	1 yr. 7 mths.	Meningococcal meningitis	0·002	2·5	0·001	1·17	0·02	25·4	Merest trace	?	0·05	62·6	—
52	2 mths.	Marasmus	0·05	9·16	0·001	0·19	0·5	89·25	0·007	1·2	0·10	20·47	Yes.
53	3 mths.	Pyloric stenosis	0·015	8·2	0·001	0·49	0·25	135	0·008	3·7	0·16	86·3	—
54	Fœtal (8 mths.)	Prematurity	0·05	9·5	0·0005	0·09	0·15	28·6	0·003	0·6	0·08	15·3	—
55	11 mths.	Meningococcal meningitis	0·01	5·3	0·001	0·05	0·06	34·4	0·003	1·6	0·05	29·16	—
56	2 yrs. 10 mths.	Acute nephritis	0·006	6·8	0·001	1·1	0·05	56·8	0·003	3·4	0·06	68·2	—
57	10 mths.	Meningococcal meningitis	0·006	3·5	0·001	0·59	0·08	44·4	0·004	2·2	0·05	28·8	—
58	8 yrs.	Pulmonary gangrene	0·01	17·5	0·0005	0·87	0·05	87·7	0·003	6·1	0·035	70·17	Yes.
59	3 wks.	Intestinal atresia	0·02	3·3	0·0005	0·09	0·45	75·7	0·004	0·7	0·12	20·19	Yes.

Table VII—(continued).

No.	Age.	Disease.	Copper.		Manganese.		Iron.		Rubidium.		Calcium.		Silver.
			% Cu dry.	Total Cu in mg.	% Mn dry.	Total Mn in mg.	% Fe dry.	Total Fe in mg.	% Rb dry.	Total Rb in mg.	% Ca dry.	Total Ca in mg.	
60	10 yrs.	Acute nephritis	0·004	6·1	0·0007	1·1	0·05	76	0·002	3·06	0·03	46	Yes.
61	7 wks.	Pyloric stenosis	0·045	9·15	0·0012	0·24	0·5	98·1	0·004	0·8	0·12	24·4	Yes.
62	3 mths.	Pylorospasm	0·04	9·1	0·0004	0·099	0·4	99	0·004	0·9	0·08	19·4	—
63	5 wks.	Broncho-pneumonia	0·05	8·6	0·0004	0·07	0·4	71·7	0·003	0·5	0·09	15·7	Yes.
64	3 wks.	Cystic hygroma	0·03	11·57	0·0002	0·079	0·35	135·6	0·003	1·2	0·08	29·9	—
65	5 wks.	Marasmus	0·04	8·2	0·0004	0·08	0·55	113·4	0·004	0·8	0·08	16·5	—
66	11 mths.	Broncho-pneumonia	0·006	3·8	0·001	0·6	0·06	38·1	0·005	2·5	0·07	45·8	Yes.
67	1 yr.	Meningococcal meningitis	0·009	7·1	0·001	0·86	0·08	68	0·004	3·2	0·10	85·9	—
68	1 mth.	Congenital heart	0·044	8·5	0·0005	0·09	0·2	41·2	0·003	0·6	0·16	32·3	Yes.
69	4 yrs. 6 mths.	Cerebral tumour	0·007	6·9	0·001	0·99	0·1	99	0·002	1·9	0·09	92	—
70	10 mths.	Intussusception	0·0075	4·4	0·0005	0·3	0·1	59·8	0·003	1·8	0·06	36·4	—
71	4 mths.	Urethral valves	0·018	4·6	0·0008	0·2	0·44	112·4	0·004	0·9	0·09	23·1	—
72	3 yrs. 2 mths.	Mastoid and empyema	0·007	8·2	0·0007	0·8	0·05	58·6	0·002	2·3	0·06	71	—
73	3 mths.	Broncho-pneumonia	0·008	3·8	0·0005	0·24	0·17	71·6	0·005	2·4	0·16	76·2	—
74	3 mths.	Prematurity	0·006	1·6	0·0002	0·05	0·06	16·2	0·004	1	0·05	13·2	—
75	10 yrs. 4 mths.	Lymphatic leukæmia	0·005	9·9	0·0005	0·9	0·15	298	0·003	5·5	0·09	176·6	—
76	8 mths.	Influenzal meningitis	0·004	3·1	0·0002	0·15	0·03	25·2	0·002	1·5	0·03	25·3	—
77	3 mths.	Marasmus	0·007	1·5	0·0005	0·10	0·13	28	0·004	0·8	0·15	31·5	Yes.
78	12 days	Prematurity	0·044	7	0·0004	0·06	0·44	68·2	0·004	0·6	+0·22	34·4	—
79	10 mths.	Intussusception	0·005	3·1	0·0014	0·91	0·1	66·1	0·004	2·28	0·12	77·5	—
80	11 mths.	Pink disease	0·007	2·9	0·0013	0·056	0·08	35·5	0·004	1·6	0·07	29·9	—
81	6 mths.	Post basic meningitis	0·015	8·4	0·0002	0·10	0·07	38·2	0·016 (Li 0·0002%)	8·6	0·05	28	—
82	11 yrs.	Tb. meningitis	0·006	11·4	0·0014	2·6	0·1	193·5	0·004	7·9	0·07	131·9	—
83	12 yrs.	Diabetic coma	0·003	8·2	0·0005	1·3	0·1	290·2	0·003	8·2	0·07	196·8	—
84	3 mths.	Marasmus	0·009	1·7	0·0003	0·06	0·3	62·1	0·004	0·8	0·05	10·6	—
85	9 yrs. 6 mths.	Rheumatic carditis	0·004	6·1	0·001	1·6	0·08	132	0·001	1·76	0·14	229	—
86	9 yrs.	Rheumatic pericarditis	0·003	4·6	0·001	1·56	0·05	78·2	0·002	3·1	0·07	109·5	—

87	8 mths.	Miliary Tb.	0·003	3·3	0·0002	0·23	0·05	57·6	Merest trace	?	0·07	78·9		—
88	9 mths.	Gastro-enteritis	0·0035	1·18	0·0012	0·4	0·22	80	0·003	1	0·12	44·4		?
89	7 wks.	Broncho-pneumonia	0·005	2·1	0·0005	0·2	0·13	57·9	0·004	1·79	0·07	30·8		—
90	4 mths.	Congenital heart	0·054	17·6	0·0002	0·06	0·04	13·1	0·004	1·25	0·08	25·1	Yes.	
91	7 mths.	Pneumonia and empyema	0·018	6·5	0·0005	0·16	0·16	54·3	0·008	2·6	0·08	26·3		—
92	1 yr. 5 mths.	Tb. meningitis	0·018	9·8	0·001	0·05	0·05	26·1	0·004	2·1	0·05	26·1	Yes.	
93	5 yrs. 7 mths.	Nephritis (mixed)	0·002	2·7	0·0008	1·08	0·18	244	Merest trace	?	0·16	203·8	Yes.	
94	1 yr. 3 mths.	Broncho-pneumonia	0·01	5·8	0·001	0·6	0·1	66·9	Merest trace	?	0·08	52·3	Yes.	
95	9 mths.	Broncho-pneumonia	0·02	14·8	0·0002	0·15	0·1	74·1	0·002	1·48	0·05	38·5		?
96	5 mths.	Tb. meningitis	0·015	5·3	0·0004	0·14	0·28	99·7	0·002	0·7	0·18	60	Yes.	
97	2 yrs.	Tb. meningitis	0·0045	3·4	0·0007	0·5	0·03	23·6	0·004	3	0·05	37·6	Yes.	
98	3 yrs. 7 mths.	Suprarenal sarcoma	0·002	2·5	0·0007	0·8	0·12	142·8	0·002	2·48	0·07	80·7		—
99	11 mths.	Broncho-pneumonia and asthma	0·004	1·6	0·0003	0·13	0·02	8·7	0·003	1·3	0·06	26	Yes.	
100	2 mths.	Gastro-enteritis	0·018	5·4	0·0002	0·06	0·32	103·1	0·004	1·3	0·16	51·04	Yes.	
101	9 yrs.	Healthy (accident)	0·01	18·5	0·0012	2·24	0·07	131·24	0·004	6·9	—	—		—
102	1 yr. 2 mths.	Pink disease	0·01	8	0·001	0·9	0·05	40·4	0·006	5·4	—	—	Yes.	
103	10 yrs.	Tb. meningitis	0·02	39·9	0·001	1·9	0·06	116·6	0·004	7·6	—	—		—
104	1 yr. 6 mths.	Empyema	0·002	1·7	0·0008	0·7	0·03	28·48	Merest trace	?	—	—		—
105	1 yr. 2 mths.	Tb. meningitis	0·002	1·8	0·0002	0·18	0·04	36·2	0·005	4·8	—	—	Yes.	
106	6 yrs.	Lymphatic leukæmia	0·009	8·7	0·0008	0·74	0·03	24·9	Merest trace	?	—	—		—
107	1 wk.	B. Coli meningitis	0·015	—	0·0008	?	0·35	?	0·002	?	—	—		—
108	10 mths.	Acute nephritis	0·0042	2·2	0·0008	0·41	0·15	77·2	0·002	1	—	—		—
109	4 yrs.	Pneumococcal meningitis	0·004	—	0·0008	?	0·035	?	0·002	?	—	—		—
110	7 mths.	Tb. meningitis	0·032	15·7	0·0008	0·39	0·32	157	0·002	0·98	—	—		—
111	5 yrs.	Empyema	0·008	—	0·0008	?	0·03	?	—	—	—	—		—

Table VIII.

No.	Age.	Copper.		Manganese.		Iron.		Rubidium.		Silver.
		% Cu dry.	Total Cu in mg.	% Mn dry.	Total Mn in mg.	% Fe dry.	Total Fe in mg.	% Rb dry.	Total Rb in mg.	
F 1. G.B.	Full-term fœtus	0·008	—	0·0016	—	0·09	—	0·0016	?	—
F 1. Liver		0·022	5·7	—0·0008	0·21	0·32	86·6	0·003	0·83	—
F 2. G.B.	Full-term fœtus	0·015	—	0·0008	—	0·16	—	0·003	?	—
F 2. Liver		0·014	3·7	—0·0008	0·19	0·25	60·1	0·0025	0·6	—
F 3. G.B.	22 weeks	0·005	—	—	—	—	—	—	—	—
F 3. Liver		0·017	0·4	Trace	?	0·22	8·9	0·004	0·14	Yes
F 4. G.B.	Full-term fœtus	0·015	—	0·002	—	0·13	—	0·003	?	Yes
F 4. Liver		0·040	9·6	0·0008	0·20	+0·20	51·1	0·004	1	Yes
F 5. G.B.	Full-term fœtus	0·015	—	0·001	—	0·13	—	0·005	—	Yes
F 5. Liver		0·021	5·8	—0·0008	0·23	0·30	88·4	0·005	1·47	—
F 6. G.B.	26 weeks	0·003	—	Trace	—	0·07	—	0·004	—	—
F 6. Liver		0·020	3	Trace	—	0·30	45·6	0·006	0·86	—
F 7. G.B.	Full-term fœtus	0·017	—	0·0025	—	0·04	—	0·003	—	—
F 7. Liver		0·045	13·5	0·002	0·6	0·20	60·6	0·004	1·19	—
F 8. G.B.	24 weeks	0·008	—	Trace	—	0·04	—	0·0016	—	—
F 8. Liver		0·020	1·28	Trace	?	0·10	6·2	0·0016	0·1	—
F 9. G.B.	30 weeks	0·016	—	None	—	0·03	—	0·0016	—	—
F 9. Liver		0·033	2·68	Trace	?	0·12	9·8	Trace	?	—
F 10. G.B.	22 weeks	0·008	—	Trace	—	0·04	—	0·0016	—	—
F 10. Liver		0·028	2·17	Trace	?	0·19	14·9	Trace	?	Yes
F 11. G.B.	Full-term fœtus	0·014	—	0·0006	—	0·16	—	0·0016	—	Yes
F 11. Liver		0·021	7·8	0·0008	0·29	0·25	93	0·0016	0·59	Yes
F 12. Head	5 cm. long	0·003	—	Merest trace	—	<0·03	—	0·004	—	—
F 12. Body without liver		0·002	—	Merest trace	—	<0·03	—	0·003	—	—
F 12. Liver		0·018	0·007	Trace	?	0·40	0·15	0·002	—	—
F 13. G.B.	24 weeks	—	—	—	—	—	—	—	—	—
F 13. Liver		0·014	0·019	Merest trace	?	0·05	0·7	—	—	—
F 14. G.B.	7·5 cm. long	—	—	—	—	—	—	—	—	—
F 14. Liver		0·013	0·004	Trace	?	0·19	0·08	0·002	—	Yes

to ascertain, there is no record of any previous investigation into the metallic content of the liver.

Copper was 0·005%, which is slightly less than half the average value for this age (0·011%). This finding contrasts with the usual state of affairs in the cirrhosis of adults, where an increase in the copper content is almost invariable. In 10 cases of adult cirrhosis, Schonheimer and Herkel (1930) found an average of 0·01% of copper which is some four times the average adult figure.

Manganese.—This occurred in very small amount (0·0001%).

Iron gave a surprising result. The lines were so faint that they would not allow of an estimation of the quantity within the limits of the present method and they have therefore been recorded as showing "traces." This is the only instance in the 125 specimens where iron is too low for estimation. This finding, as for copper, is in contrast to adult cirrhosis, where the amount of iron is usually increased (Kretz, 1896). The importance of such a deficient pre-natal store of iron and copper is to be seen from the fact that the child died at the age of 8 months, with a profound anæmia (Hb = 15%, C.I. = 0·4).

Rubidium.—This metal was low (0·002%).

Calcium.—This metal was low (0·03%).

It is clear that in congenital cirrhosis, in contrast to the adult type of the disease, the liver shows a striking deficiency in its metallic content, which applies especially to iron. It would appear that the cirrhosis prevents the liver from exercising its natural function in fœtal life of storing iron and copper, and even where there is normally no pre-natal store of a metal it is laid down in deficient amount.

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Summary and Conclusions.

The results of spectrographic analysis of the livers from 111 children and 14 fœtuses are recorded.

Iron.—The analyses confirm the original finding of Bunge that iron is stored in the liver during fœtal life, while the recent observation of Gladstone that

iron continues to be stored in the liver for 2 months after birth owing to the physiological post-natal hæmolysis, is also confirmed. After that period the amount of iron falls rapidly (continuing in our series until the end of the second year) throughout the nursing period, to rise again when a mixed diet is taken. Analysis of fœtal gall-bladders indicates that iron is excreted from the liver during fœtal life, but since the metal is almost entirely absent from the meconium it would appear that there is reabsorption of iron from the gut.

Copper.—Copper is also stored in the liver during fœtal life. During the nursing period, the concentration of copper falls, rising again at the end of this period, when the child is weaned on to a mixed diet. It is probable that the fœtus derives actually more copper from the placenta than it needs, since this metal occurs in the fœtal gall-bladder and in meconium.

Iron, Copper and Calcium.—There is a close parallel between the relative amounts of these three metals in the livers of children. The parallelism between iron and copper is probably attributable to their co-operation in the synthesis of hæmoglobin, and it is suggested that for the optimum absorption of these metals, an adequate supply of calcium is necessary.

Manganese.—The percentage of manganese in the fœtal liver rises during the last 3 months of intra-uterine development. The amount decreases during the nursing period, to rise again when a mixed diet is given. The analyses show that there is no storage of manganese during fœtal life, and in this respect manganese differs from both iron and copper in which the birth level is not regained. There is an active excretion of manganese from the liver by way of the gall-bladder and meconium during the last months of fœtal life.

Rubidium.—Rubidium is not stored during intra-uterine life. During the nursing period there is a storage of rubidium, but the significance of this is not understood.

Rubidium-Potassium.—A rough parallelism was noted between the stored amounts of these two metals, possibly related to their close chemical affinity.

Silver.—The presence of silver in traces was noted in roughly a third of the cases.

A study of the results of the analyses in relation to the diseases causing death revealed connections in several instances. One of the most consistent features was the high content of rubidium and other metals in the livers of infants dying from pyloric stenosis. A case of congenital cirrhosis of the liver was remarkable for the low copper value and almost complete absence of iron—a finding in sharp contrast to adult cirrhosis. The amounts of rubidium, calcium and manganese were also low, and the results suggest that the liver,

being diseased early in fœtal life, was unable to store the various metals to anything like the usual amount.

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The Duration of the Recovery Period following Strenuous Muscular Exercise.

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There are a variety of ways in which the duration of the recovery period after exercise can be determined. The method most frequently employed depends upon observations of the respiratory metabolism. This method has been chosen because the respiratory changes due to exercise can be followed with reasonable ease and accuracy, and because these changes are among the last of the more obvious effects of the exercise to disappear during recovery. In addition, interesting data concerning the effects of exercise on respiratory metabolism can be collected during the determination of the duration of the recovery period when this method is used.

In determining the duration of the recovery period by observation of the respiratory metabolism, it is necessary to decide when the carbon dioxide output and oxygen intake have returned to their normal values and are no longer affected by the process of recovery from the exercise. This decision has been made in a variety of ways by different investigators. Some have made one or more pre-exercise determinations of the subject's basal oxygen intake and carbon dioxide output. Recovery was said to be complete when

the carbon dioxide output and oxygen consumption returned to these values after exercise. Others found that the oxygen consumption did not return to the pre-exercise level within a reasonable length of time, but remained above normal for several hours. They considered that recovery was complete when the carbon dioxide output and oxygen intake returned to a steady level after exercise, even if the level was not the same as that before exercise.

Neither of the foregoing methods of deciding upon the duration of the recovery process takes any account of the normal variation of the basal carbon dioxide output and oxygen intake, from hour to hour, in a given subject. The normal range of variation of the basal values is quite considerable, even in a well-trained subject. It is obviously unwise to assume that the results of one or even two short collections accurately represent the average basal metabolism of the subject. In the present investigation an attempt has been made to obviate the necessity for making this assumption. For this purpose, the normal range of the basal carbon dioxide output and oxygen consumption has been established for each subject by means of several long series of basal collections. Recovery is then said to be complete when the carbon dioxide output and oxygen intake return within these normal limits after exercise. In those subjects who show a prolonged increase in the oxygen intake after exercise, recovery is said to be complete when the oxygen intake no longer shows a wider range of variation than is observed in the basal state. Recovery, defined in this way, was apparently complete in the three subjects studied, in less than 1½ hours after 30 seconds of strenuous exercise.

Apparatus and Technique.

A slight modification of the ordinary Douglas bag technique was used for the determinations of carbon dioxide output and oxygen intake. The Douglas bag method is simple and widely used, but nevertheless entails certain possibilities of serious error. These possibilities have been reviewed by Hill, Long and Lupton (1924, *b*). In some preliminary experiments it was found that the air metered into a Douglas bag could not be consistently recovered with a reasonable degree of accuracy, especially from the larger bags. The cause of the discrepancies was not ascertained. However, it was apparent that the solution of the difficulty lay in metering the air before it was collected in the bags. With this purpose in mind, the apparatus shown diagrammatically in fig. 1 was assembled.

As shown in the diagram, the air expired by the subject is first collected in

a 10-litre spirometer. From the spirometer it is pumped through a cooling coil, through the meter, and then into a Douglas bag. In this way the air is metered before entering the bag, and the bag is only used to collect the air so that a sample may be taken.

The valves used were of the conventional type, consisting of a circular rubber flap, seating on a metal ring. They were tested before each experiment. The pump used was of the movable blade rotary type and was driven by an electric motor. The meter used was a Sargent wet test gas meter. This type of meter is very sensitive to changes in water level, as Krogh (1920) has pointed out, hence the water level was checked before each experiment. The correct

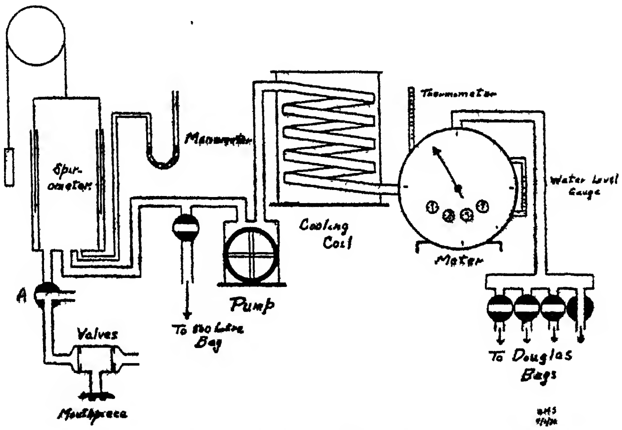

FIG. 1.—Apparatus for metering and collecting respired air.

water level was determined by checking the meter against a small Bohr meter which had been calibrated by displacement of air. The accuracy of the Sargent meter was guaranteed by the makers to within 1 per cent. at rates of flow up to 50 cubic feet per hour. During the experiments the rate of metering was never allowed to exceed this value. The cooling coil was used to prevent too great a rise in meter temperature owing to passage of warm expired air through the meter. The effectiveness of this device is shown by the fact that the meter temperature seldom changed by as much as one degree during an experiment (2 to 3 hours). By cooling the air, complete saturation with water vapour was also ensured.

The whole apparatus was periodically tested for leaks by means of a pressure pump and manometer.

The method of using the apparatus is as follows. While the subject is resting with nose-clip and mouth-piece in place, valve A, is turned to connect

the subject to the spirometer. He is allowed to breathe thus for at least 15 minutes. The expired air is periodically pumped from the spirometer, through the meter, and into the Douglas bags. The bags are thoroughly rinsed with expired air and rolled to expel the air as completely as possible. A small amount of residual air can be neglected since it will change the composition of the expired air, collected later in the bag, little, if at all. Then valve A is turned to connect the subject to the atmosphere. The pump is now turned slowly by hand until the manometer indicates a negative pressure of $\frac{1}{2}$ cm. of water in the spirometer. The pointers on the meter are then set to zero and everything is ready for the first collection.

In making a collection, valve A is turned to the spirometer and the time taken. At the end of the required time, the valve is again turned to the atmosphere, the spirometer pumped out as before and the meter reading taken. The meter temperature is recorded at intervals throughout the collection.

Since the meter cannot handle the increased ventilation for a few minutes after exercise, it is necessary to replace the spirometer with a larger reservoir for the exercise experiments. For this purpose the spirometer is put out of action by placing a weight on the bell and its place is taken by a 500-litre Douglas bag connected as shown in fig. 1. At the end of the collection this bag is pumped out along with the spirometer.

The obvious defect in this apparatus is the large dead space, especially in the spirometer. This fault was overcome as far as possible by thoroughly rinsing the whole apparatus, including the bags, with expired air before starting an experiment, and by pumping out the spirometer and connecting tubes to a negative pressure of $\frac{1}{2}$ cm. of water before and after each collection, as has been described.

The advantages of the method are numerous. It makes possible the direct metering of the expired air at an approximately constant temperature and a fairly steady rate of flow. There is greater ease of sampling because there is no need of avoiding loss of air during the taking of samples. Also, the samples can be taken as soon as the collection terminates, thus avoiding loss of carbon dioxide by diffusion through the bag. This method of collection is especially convenient when collections are made in the 1000-litre cubical Douglas bag. Bags of this size and type are awkward to handle when full and are very hard to empty by rolling. One skilful person has no difficulty in doing the timing, metering, collecting, mixing, sampling, recording of the meter temperature and emptying the bags, even when using the largest bags.

After thorough mixing, duplicate samples were taken from each bag in

Brodie sampling tubes. The samples were analysed in duplicate on ordinary sets of Haldane-Henderson gas analysis apparatus. In almost every experiment the two analyses for any one collection were done from different sampling tubes, on different Haldanes and by different experimenters. About 66% of the carbon dioxide and 69% of the oxygen analyses show an absolute difference of 0·03% or less between duplicates. About 98% of all the analyses showed a difference of 0·05% or less. The greatest difference between duplicates was 0·07%. An absolute difference of 0·05% corresponds to a percentage difference of less than one-third of 1% on an oxygen analysis and just over 1% on a carbon dioxide analysis.

When using the Douglas bag technique, the percentage of carbon dioxide in the inspired air must be known. In preliminary experiments it was found that a carbon dioxide content of 0·1% or more might occur in the inspired air if anyone was working near the subject. To avoid such a high carbon dioxide content, a large fan was arranged to send a draught over the opening of the inlet valve. Air samples taken continuously over a period of about 45 minutes, in the region of the air intake, showed a carbon dioxide content of 0·04% to 0·05% with the fan in operation. The carbon dioxide content of the inspired air was taken as 0·04% for all the experiments.

Experiments.

All the experiments were performed in the morning. The subject came without his breakfast so that he could attain the strictly " basal " state. Two types of experiments were performed. The first type might be called " basal controls." In these the subject's basal carbon dioxide output and oxygen consumption were determined over a period of 1½ to 2½ hours. The expired air was collected in a series of 13-minute periods with 2 minute intervals between them for pumping out the spirometer and reading the meter.

The second type was the exercise experiments. In these the subject first rested for 30 minutes or more. Then one 13-minute basal collection was taken. The exercise was then performed. The expired air was collected during the exercise, and for ½ to 1¼ hours afterward, in one large bag. Following this, collection was resumed in 13-minute periods as in the " basal controls ". These collections were continued until 2 to 3 hours after the exercise.

The exercise performed was standing running at top speed. The subject ran until he could no longer continue running at a rapid rate. This stage was

reached in from 30 to 45 seconds. The exercise may be classed as strenuous, as the high oxygen requirements indicate.

"Basal Controls."

As mentioned in the introduction, an attempt was made in this investigation to determine the normal range of variation of the basal carbon dioxide output and oxygen intake in each subject studied. For this purpose several "basal control" experiments were performed on each subject. Six to eight 13-minute collections were made in each series. These collections were arranged to cover approximately the same hours of the day as the exercise experiments, so that the two would be strictly comparable.

The amount of variation shown in these experiments proves that their performance was justified. The extreme range between the highest and the lowest results for each subject is shown in Table I. It could certainly be argued that recovery is complete following exercise when the carbon dioxide output and oxygen intake return within, and stay within, these limits. However, each limit thus defined depends on the result of a single observation. It would probably be more accurate to limit the normal range above, by the

Table I.

Subject.	CO_2.			O_2.		
	Highest.	Lowest.	Difference.	Highest.	Lowest.	Difference.
T. B.	223	174	49	257	218	39
C. H. B.	198	174	24	248	219	29
A. L. C.	229	191	38	281	234	47

All figures in cubic centimetres per minute.

average of the highest values found in each series, and below, by the average of the lowest values found in each series. Those values are shown in Table II and also appear as the normal limits of the basal range on the graphs of the exercise experiments. The standard deviation of an individual measurement might also be used to define the upper and lower limits of the normal range. The use of the standard deviation to limit the normal range would not materially alter the conclusions reached. Table III shows the average carbon dioxide output and oxygen intake, together with the standard deviation of an individual measurement, and of the average for all the basal control collections on each subject.

Table II.—(Carbon dioxide outputs and oxygen consumptions in cubic centimetres per minute.)

Subject.	No. of series.	No. of collections.	CO_2.				O_2.				R.Q.			
			Average.	Average high.	Average low.	Difference.	Average.	Average high.	Average low.	Difference.	Average.	Average high.	Average low.	Difference.
T. B.	4	30	201	217	185	32	238	254	226	28	0·84	0·88	0·81	0·07
C. H. B.	3	22	187	194	180	14	235	242	226	16	0·80	0·81	0·77	0·04
A. L. C.	4	29	212	224	198	26	263	276	246	30	0·80	0·84	0·77	0·07

Table III.

(Carbon dioxide outputs and oxygen intakes in cubic centimetres per minute.)

Subject.	No. of collections.	Carbon dioxide.			Oxygen.		
		Average.	S.D. of individual measurements.	S.D. of average.	Average.	S.D. of individual measurements.	S.D. of average.
T. B.	30	201	±13·4	±2·44	238	±10·5	±1·91
C. H. B.	22	187	±6·2	±1·33	234	±8·7	±1·85
A. L. C.	29	212	±8·8	±1·63	263	±10·9	±2·03

The variations shown in these tables, especially Table I, seem very large at first sight. In Table IV are shown the basal metabolic rates calculated for the highest and the lowest oxygen consumptions recorded for each subject. This table shows that none of the subjects exhibited a greater range of B.M.R. than the allowable 20%.

Table IV.

Subject.	Average B.M.R. per cent.	Highest B.M.R. per cent.	Lowest B.M.R. per cent.
T. B.	− 4·6	+ 1·7	−12·6
C. H. B.	−19	−14	−24
A. L. C.	+ 1·9	+ 8·9	− 8·6

It is interesting to note that subject C. H. B., who has a much less variable B.M.R. than either of the other subjects, appeared to be much more restless than they were. The other two subjects both complained of cramps and general stiffness after lying in one position for a long time. This discomfort would undoubtedly raise their B.M.R. C. H. B. avoided discomfort, as far as possible, by slight movements when necessary. It is very likely that the low B.M.R. shown by this subject is due, in part at least, to his ability to relax. It is noticeable that a new subject frequently shows a higher B.M.R. at first than he does later when he has become accustomed to the procedure. This high initial B.M.R. is not necessarily associated with restlessness. The new subject is often so impressed with the need for remaining motionless that he becomes quite tense. Later when he learns to relax and to move slightly, when necessary to relieve discomfort, his B.M.R. falls.

Duration of the Recovery Period.

The carbon dioxide outputs and oxygen consumptions for the exercise experiments were plotted against time. The resulting curves are shown in figs. 2, 3 and 4. On these curves are marked the limits of the normal basal range. These limits are the values designated "average high" and "average low" in Table II. In certain cases the highest or lowest basal value is also indicated. Recovery is considered to be complete when both the carbon dioxide output and the oxygen intake return within, and remain within, these normal limits.

The necessity for establishing the normal range of variation of the basal metabolism in each subject is made clear by a study of the graphs. As shown in figs. 2, 3 and 4 the pre-exercise level of the carbon dioxide output is below the normal range in five, and the oxygen intake is below the normal range in six, out of the ten experiments. The cause of these low pre-exercise basal values is not known, but they are apparently due to anticipation of the exercise, since there is no indication of similarly low values in the first collections of the basal series. Had these pre-exercise collections been assumed to represent the true level of the basal metabolism recovery would have appeared to be far from complete even at the end of 2½ hours.

The results of the four exercise experiments on subject T. B. are plotted in fig. 2. It is clear that in experiments 2 and 3 the carbon dioxide output is back within normal limits in 1 hour. In experiments 1 and 4 it does not come back within the limits until 1½ hours after exercise. In experiment 1 there is no tendency for the carbon dioxide output to leave the normal range. In the other three experiments there is a definite upward tendency toward the end of the experiments, but it seems unlikely that the carbon dioxide output would again go above the normal range after having remained within the range for 1 hour, as it did in experiments 2 and 3.

The oxygen consumption shows more rapid recovery. In experiment 2 it is back within the normal limits in less than three-quarters of an hour, in experiment 4 in less than an hour and in experiments 1 and 3 in less than 1½ hours. In none of the experiments is there any tendency for the oxygen intake to leave the normal limits again.

The results of the experiments on C. H. B. are not so clear-cut. In experiment 5 there is slight carbon dioxide retention up to the end of collections at 2 hours. In experiment 6 the carbon dioxide returns within the normal range in about 1½ hours. It leaves the normal range once between 1¾ and 2 hours, but does

not fall below the lowest observed basal value. It seems safe to say that recovery is complete in this experiment, as far as carbon dioxide is concerned, in 1½ hours. Experiment 7 shows the carbon dioxide output returning within the normal range in less than 1½ hours, and remaining there.

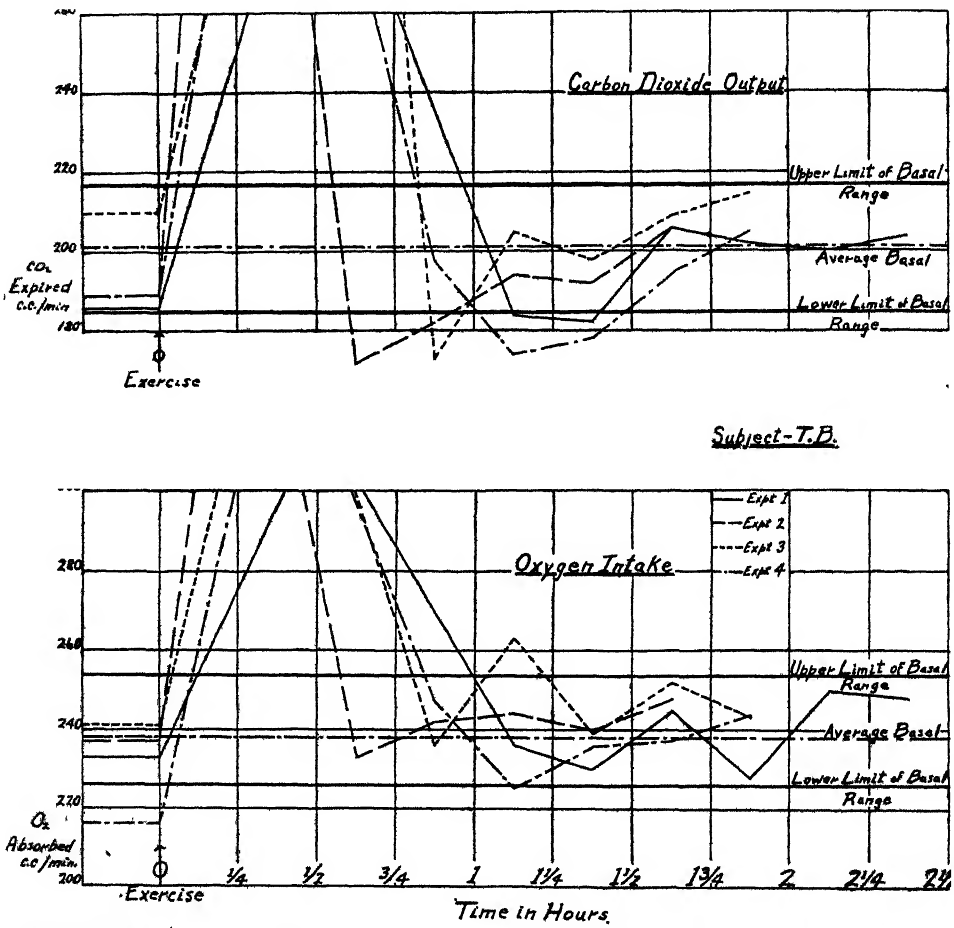

FIG. 2.—Carbon dioxide output and oxygen intake curves for exercise experiments on subject T. B.

In all three experiments on C. H. B. the oxygen intake seems to have attained its normal constancy within less than 1 hour after exercise, but it varies through a range which is slightly above the normal basal range. In only one experiment (No. 5) does it remain within the normal limits for any considerable period and in that case it is confined to the upper part of the basal range. It is to be noted that the normal basal range in this subject is considerably less than

in the other subjects, and that the range covered by the carbon dioxide output and oxygen intake after exercise is similarly reduced.

In experiments 8 and 9 on A. L. C. the carbon dioxide output returns within normal limits within $1\frac{1}{2}$ hours and does not leave the normal range again

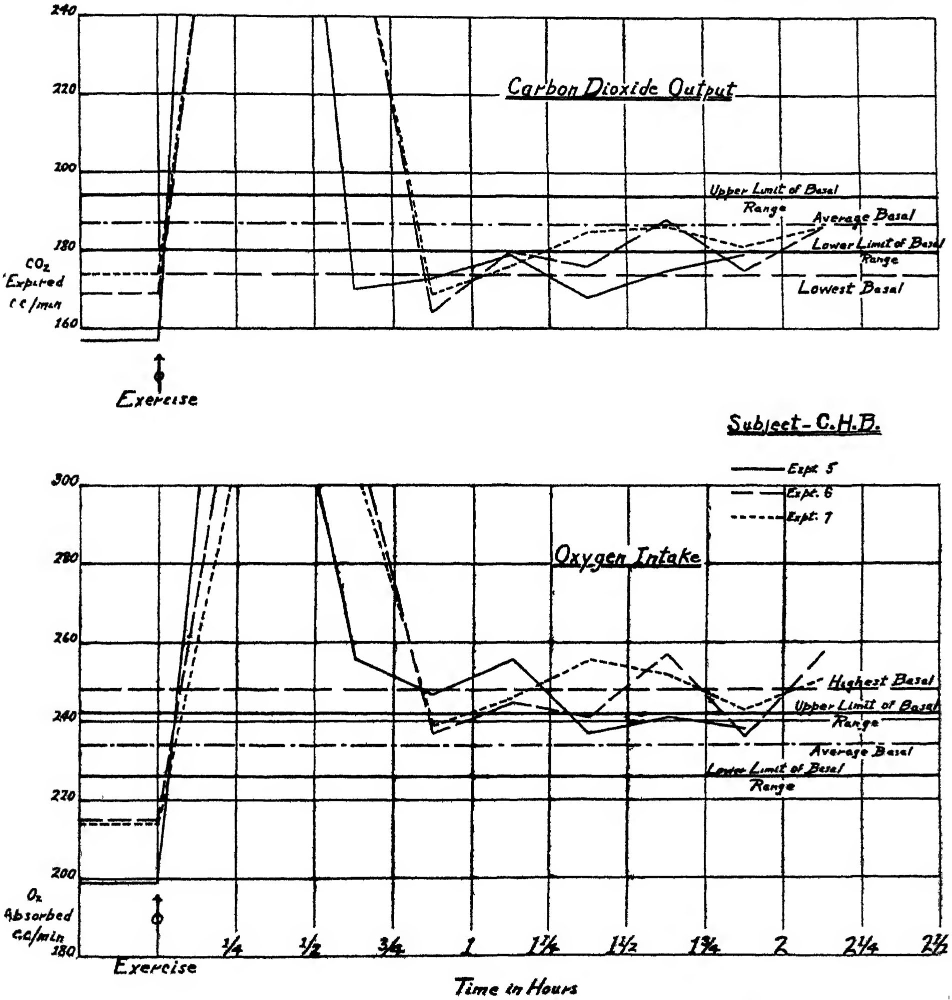

Fig. 3.—Carbon dioxide output and oxygen intake curves for exercise experiments on subject C. H. B.

although the collections in experiment 8 were continued until $2\frac{3}{4}$ hours after the exercise. In experiment 10 the carbon dioxide output shows a slight drop below the normal limit between $1\frac{1}{2}$ and $1\frac{3}{4}$ hours after exercise but remains within the normal limits thereafter. This low point is not below the lowest observed basal value, so its significance is doubtful.

In all three experiments on A. L. C. the oxygen intake returns to a range which is definitely above the normal basal range. It appears to have attained a normal constancy in all the experiments in about 1½ hours.

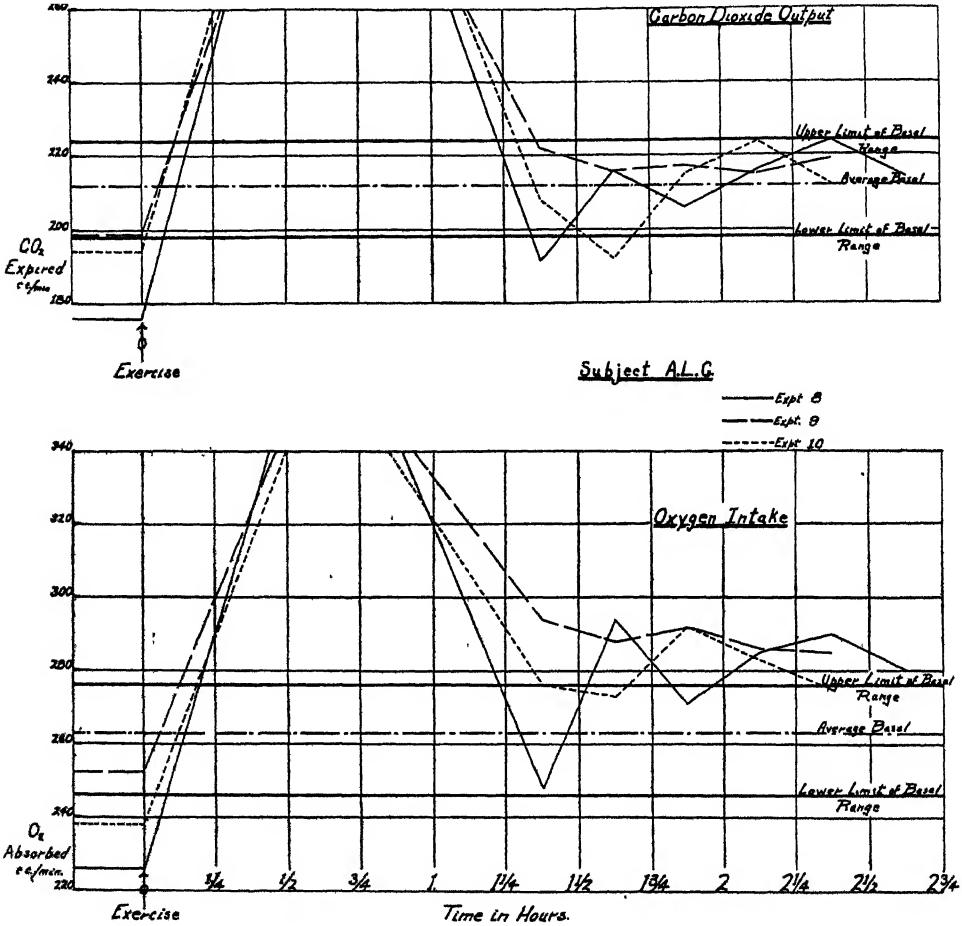

FIG. 4.—Carbon dioxide output and oxygen intake curves for exercise experiments on subject A. L. C.

Discussion.

In the four experiments on subject T. B. there seems to be no doubt that recovery is complete, as nearly as can be judged by present methods of studying respiratory metabolism, in less than 1½ hours. In the experiments on the other two subjects the interpretation of the results is complicated by the prolonged rise in oxygen intake which follows the exercise. This increase in the level of

the resting oxygen intake occurs in every one of the six experiments performed on these two subjects, and in no case has it disappeared by the end of the period of collection. In C. H. B. the average level of oxygen intake after exercise is about 6·4% above the average basal level as shown by the "basal control" experiments. In A. L. C. the corresponding increase is about 8%.

This prolonged increase in oxygen consumption following strenuous exercise of short duration, has been reported before. The results obtained on two of the three subjects studied in the present investigation are in complete agreement with the results reported by Hill, Long and Lupton (1924, *c*). These workers state that, "after severe exercise the resting oxygen intake returns in about 80 minutes to a constant level which is some 7% higher than it was before exercise." Sargent (1926) also observed a 7% rise in the resting oxygen consumption after exercise. Gemmill (1931) obtained a similar rise in one experiment. In his four other experiments the oxygen intake returned to the resting level within 2 hours. He concludes that "work produces no change in the demand for oxygen which is not returned completely to the previous basal state during the recovery period." The present experiments seem to indicate that a prolonged increase in the oxygen intake after exercise is characteristic of certain subjects, while other subjects under identical conditions may show a rapid return of the oxygen intake to its resting level.

Hill, Long and Lupton (1924, *c*) state that "This rise in basal metabolism is not a part of the recovery process, but an effect of the general circulatory and metabolic disturbance produced by exercise." Sargent also subscribes to this view. Since there is little available evidence for or against Hill's statement it can tentatively be accepted. If this shift in the level of the oxygen intake is not due to the recovery process, then it is fairly safe to say that recovery was complete in all the subjects observed in less than 1½ hours after a short bout of strenuous exercise.

In a few experiments, and especially in experiment 5, there is a suggestion that there may still be a slight retention of carbon dioxide occurring even 2½ hours after exercise. It hardly seems feasible, with the present methods of studying the gaseous exchange, to rule out the possibility, or prove the occurrence, of a small and prolonged carbon dioxide retention after exercise. The observation of Liljestrand and Wilson (1925) that the kidney may be retaining base several hours after a short period of exercise, suggests the possibility of a similar retention of carbon dioxide.

A great many workers have made observations on the duration of the recovery period following exercise of widely varying duration and intensity. Only the work of those who studied the effect of very strenuous exercise of short duration need be mentioned here. Hill, Long and Lupton (1924, *a*) observed the effects of several types of exercise, including standing running at high speeds for periods of less than 30 seconds. They concluded that "the process of recovery appears to be complete, after the most strenuous exertion, in about 90 minutes." This conclusion was based on a study of the R.Q. and the blood lactic acid after exercise. In some of his experiments Sargent used standing running "all out" for 30 seconds as the exercise. From observations of the oxygen intake alone he concluded that recovery was complete in all but one case in less than 20 minutes. The oxygen intake may have returned to normal with equal rapidity in some of the present experiments, but the taking of long collections immediately after the exercise makes it impossible to obtain any data on the period in question.

Gemmill had his subjects run 60 to 70 yards in 10 seconds on a treadmill. He concludes that the oxygen intake returns to its resting level within two hours after exercise and the carbon dioxide output within three. When due allowance is made for the normal variability of the basal metabolism, it seems certain that recovery was complete much sooner than this. A study of his figures does not reveal any reason for saying that recovery is complete in 3 hours rather than in 1½ hours. It would appear that he places too much reliance upon the result of the pre-exercise basal determinations.

It seems probable that recovery, as defined in the present paper, was complete in Gemmill's subjects in at least as short a time as in the subjects used in this investigation. There is a remarkable similarity in the experimental results of the two investigations. The great divergence of the conclusions reached seems to depend upon the definition of recovery.

The period of carbon dioxide retention which occurs following strenuous exercise of short duration is interesting. Hill, Long and Lupton (1924, *a*; 1924, *c*) reported it in their experiments. Gemmill reports not only the initial period of carbon dioxide retention, but also a second one, occurring later. The present experiments do not show the retention very clearly because of the prolonged initial collection after exercise. However, there is no doubt that the retention did occur, as can be seen from figs. 2, 3 and 4.

In general, it would appear that the oxygen intake returns to a steady level, after strenuous exercise of short duration, fairly rapidly, and certainly in less than 1½ hours. The course of the carbon dioxide output after exercise is

complicated by the disturbance of the acid-base balance of the body caused by the exercise. During and immediately after exercise, there is over-ventilation and loss of pre-formed carbon dioxide. This loss is later made good by carbon dioxide retention so that the acid-base balance is restored to its former level. The acid-base situation in exercise is further complicated by the compensatory activities of the kidneys. This carbon dioxide retention is more prolonged than the effect of the exercise on the oxygen intake, but also appears to be complete within 1½ hours after a short period of standing running.

The Respiratory Quotient of the Excess Metabolism.

There is a growing body of evidence which suggests that the classical interpretations of the respiratory quotient are based on unjustifiable assumptions. It now seems probable that a single interpretation of a respiratory quotient is rarely possible. This fact greatly lessens the significance of any measurement of the respiratory quotient, and especially measurements made during and following strenuous exercise when the metabolism is greatly disturbed and transformations of foodstuffs may be occurring. However, since the determinations of the duration of the recovery period, which have already been described, yield all the data necessary for the calculation of the respiratory quotient of the excess metabolism following strenuous exercise of short duration, it seems worth while to add these data to the literature on the subject.

In order to obtain reasonably reliable values for the excess metabolism following strenuous exercise, care must be taken that the metabolism is followed until recovery is complete and that a reliable base line is chosen. The evidence that recovery was followed to completion in the present experiments has been presented. In the experiments on subject T. B., in all of which the oxygen intake returned rapidly to its resting level after exercise, the average values of oxygen intake and carbon dioxide output, obtained from the basal control experiments, unquestionably supply the best base line from which to calculate the excess metabolism. In the experiments on subjects C. H. B. and A. L. C. the carbon dioxide base line can be similarly obtained. With these subjects there are two oxygen base lines which might be chosen, first, the average value of the oxygen intake obtained from the basal control experiments, and second, the average value of the oxygen intake after it has returned to a steady level following exercise. If it is tentatively accepted that the shift in oxygen intake, which is observed following exercise, is not a part of the recovery process, it is obviously more consistent to make use of the post-

exercise level as a base line. Table V shows the respiratory quotients of the excess metabolism for the exercise experiments calculated using the base lines obtained from the basal control experiments. Table VI shows the excess respiratory quotients for the experiments on subjects C. H. B. and A. L. C., calculated using the carbon dioxide base line from the basal control experiments and the post-exercise oxygen base line.

Table V.

Subject.	Experiment No.	1½ hours recovery.		2½ hours recovery.*	
		Excess R.Q.	Excess O_2 in litres.	Excess R.Q.	Excess O_2 in litres.
T. B.	1	1·32	4·74	1·27	5·02
	2	1·31	4·40	1·29	4·54
	3	1·35	4·82	1·32	4·89
	4	1·52	5·94	1·52	6·22
C. H. B.	5	1·37	5·67	1·29	5·80
	6	1·14	7·29	1·02	7·98
	7	1·26	6·44	1·14	7·05
A. L. C.	8	1·15	9·30	1·03	10·62
	9	1·10	8·64	0·97	10·12
	10	1·36	8·00	1·03	9·06

* In columns headed 2½ hours recovery, experiment 2 is calculated for 1¾ hours recovery, experiments 3, 4 and 5 for 2 hours, and experiments 6 and 7 for 2¼ hours.

Table VI.

Subject.	Experiment No.	1½ hours recovery.		2½ hours recovery.	
		Excess R.Q.	Excess O_2 in litres.	Excess R.Q.	Excess O_2 in litres.
C. H. B.	6	1·40	5·94	1·36	5·96
	7	1·60	5·08	1·60	5·02
A. L. C.	8	1·44	7·41	1·46	7·47
	9	1·41	6·75	1·41	6·98
	10	1·78	6·10	1·66	5·91

In columns headed 2½ hours, recovery experiments 6 and 7 are calculated for only 2¼ hours recovery.

Experiments 6 and 7 are calculated with base lines, CO_2, 187 c.c./min.; O_2, 250 c.c./min.

Experiments 8, 9 and 10 with base lines, CO_2, 212 c.c./min.; O_2, 284 c.c./min.

Experiment 5 was not included in this table because the shift in oxygen base line was much less marked in it than in the other experiments.

The results obtained on subject T. B. seem to show conclusively that the respiratory quotient of the excess metabolism due to strenuous exercise of short duration and complete recovery therefrom is definitely greater than unity, even when recovery is quite complete as far as measurable respiratory changes are concerned. The cause of these high respiratory quotients is not known. Of the many possible causes, two important ones at once suggest themselves. (1) The high respiratory quotient may result from a loss of pre-formed carbon dioxide with a resultant decrease of the carbon dioxide reserves of the body, the loss being later made good by a slight but prolonged retention of carbon dioxide. The observations of Best, Furusawa and Ridout (1929) upon the effects of repeated bouts of exercise render this explanation improbable. However, it cannot be dismissed as a possibility, since the extent of the available carbon dioxide reserves of the body is not known with certainty. (2) The high respiratory quotient may result from a transformation of mobilized carbohydrate into fat. Many observations upon the rise in respiratory quotient after the ingestion of sugars lend some support to this idea. A consistent rise in blood sugar after exercise of the type used in the present experiments is also suggestive (Ferguson and Solandt, 1932). The fact that this rise in blood sugar was not seen in one subject by Best, Furusawa and Ridout does not prove that carbohydrate was not mobilized in that subject. A more complete discussion of the significance of the excess respiratory quotient is given by these workers.

Gemmill (1931) states that " a consideration of the inherent and unavoidable errors in the calculation of the 'excess respiratory quotient' shows that the excess respiratory quotient for long recovery periods is a mathematical abstraction." The present experiments seem to show that, in some subjects at least, the period of recovery after very violent exercise of short duration is so short that the excess respiratory quotient may be regarded as an experimental fact rather than a mathematical abstraction. It must be admitted that the possible errors in the measurement of the excess respiratory quotient, especially with long recovery periods, are very large. However, it is felt that the excess respiratory quotients for subject T. B. have been evaluated with sufficient accuracy to warrant a further search for the cause of the high values observed.

Bierring (1932) objects to the performance of experiments where the work period is of short duration and recovery must be included, on the grounds that the errors are larger than when work is done in the steady state and recovery is not included. His objection is undoubtedly valid if the exercise performed is mild enough for a steady state to be maintained. However, it is of great

interest and importance to study the reactions of the body to exercise of intensities far exceeding the maximum attainable in the steady state, and for this purpose the type of experiment used in the present investigation seems to be the best available.

It is a pleasure to acknowledge our indebtedness to Dr. C. H. Best for his wise counsel during the planning and execution of these experiments, and to Mr. Campbell Cowan for his expert technical assistance.

Summary.

(1) The normal range of variation of the basal oxygen intake and carbon dioxide output has been determined for each of three subjects.

(2) Recovery has been taken to be complete, following 30 to 45 seconds of strenuous exercise, when the carbon dioxide output and oxygen intake once more return within, and stay within, these normal limits.

(3) One of the subjects showed complete recovery in less than $1\frac{1}{2}$ hours. The other two subjects showed a slight but prolonged increase in the resting oxygen consumption after exercise. Assuming that this shift in the level of the resting oxygen intake is not due to the recovery process, recovery was complete in these subjects in less than $1\frac{1}{2}$ hour after the exercise.

(4) The respiratory quotient of the excess metabolism due to strenuous exercise of short duration is over unity when determined by the methods outlined in this paper.

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The Three Phases of Nerve Heat Production.

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(Received May 2, 1933.)

The heat production of stimulated nerve occurs in two main stages, initial and recovery. The first object of this paper is to define and distinguish the initial heat more clearly; the second is to present fresh evidence that the recovery heat occurs in two phases, the first complete in a few seconds, the second only in 30 minutes.

Since an earlier paper (Hill, 1932, *a*) was written, various attempts have been made to quicken up the recording system of thermopile and galvanometer, so as to secure a sharper analysis of the heat. A new thermopile has been constructed by Mr. A. C. Downing, with soldered constantan-iron couples and bakelite insulation. It is very rapid in its action—far more rapid than the galvanometer. It is no more sensitive than the old one, but possesses the great advantage for some purposes that it is so well shielded from heat leaking from the stimulating electrodes that a correction is seldom necessary.

A single galvanometer can be used to record the heat, but if so it must be a very sensitive one and consequently slow. Amplification, therefore, with a photo-electric cell coupling two galvanometers has been employed, which introduces no lag except that of the second galvanometer. The latter must be one of short period. The photo-relay is superior to the thermal relay which introduces considerable extra lag. In most of the critical experiments a Zernicke moving coil galvanometer (Zb) of period 1·8 second was coupled by a Cu_2O photo-cell either to a Downing moving magnet galvanometer, or to a Moll galvanometer of period about 1·2 second. With this arrangement a full deflection is reached about $2\frac{1}{2}$ seconds after introducing a constant current.

At high sensitivity random disturbances, mechanical, thermal and electrical, are serious, but these have been avoided (*a*) by the use of Julius suspensions for both galvanometers, (*b*) by the closed thermostat described in the following paper, (*c*) by better insulation, and (*d*) in certain experiments by taking the mean of a large number of records.

Stimulation and all other details of technique were as described before (Hill, 1932, *a*); the nerves were from large Hungarian frogs (*R. esc.*).

The experiment shown in fig. 1 is one of the most decisive. The temperature was rather low (15·7° C.) so the nerve processes were somewhat slower—and therefore more easy to analyse—than at the usual room temperature of about 20° C. The quick galvanometer system was employed. Analysis was in blocks of heat of ¾-second duration, using a heating control of the same duration; the mean of 29 records of 9 seconds (12 units) maximal stimulation was employed; stimulation was at 460 shocks per second, at about 4-minute intervals. The results are given in Table I.

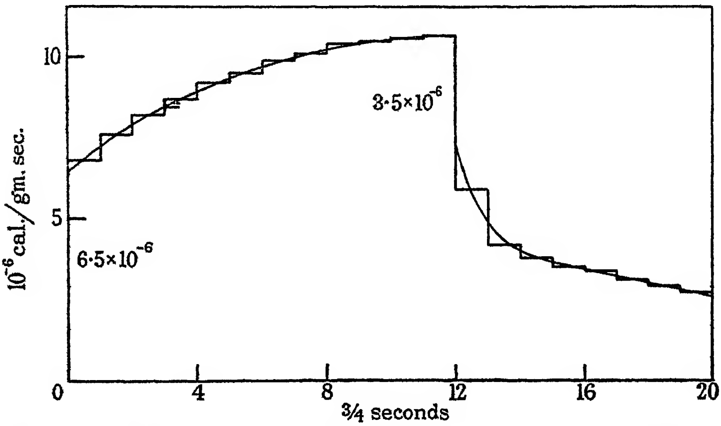

Fig. 1.—Analysis, in ¾-second time units, of heat rate during and after 9 seconds stimulation of frog's nerves at 15·7° C. See Table I and text.

By reason of the quick galvanometer system and the accuracy of the mean of 29 records, the analysis is quite determinate in spite of the short interval (¾ second) employed. The "remainders" are very small and the solution is smooth. The initial heat is sharply defined, both at the beginning and at the end of stimulation, and it is noticeable that the latter ($3{\cdot}5 \times 10^{-6}$ cal./gm. sec.) is much less than the former ($6{\cdot}5 \times 10^{-6}$ cal./gm. sec.). This "fatigue" effect is the reason why the curve tends to reach a maximum instead of continuing to rise. It is intermediate in type between curves IV and V, fig. 6.

Another example is given in fig. 2, together with the means of the records from which the analyses were made. The initial heat again is quite definite; it decreases, but not much, during stimulation. The curves can be compared with the calculated ones of fig. 4.

Fig. 3 (see Table II) represents the analysis in three different time units, 1, 2 and 4 seconds respectively, of the deflection caused by 16 seconds stimulation. Although of course the first is the most decisive the others yield

Table I.—Analysis of record of 9 seconds stimulus at 15·7° C. (See fig. 1.)

Time, ¾ second.	0.	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
Mean record	0	7	54	127	213	300	388	475	560	646	728	810	888
¾-second control	0	100	617	918	936	903	855	808	762	720	680	643	610
Solution*	75	83	91	97	102	106	110	112	115	116	117	118	66
Remainder	0	0	0	—2	1	1	1	0	—1	—1	—1	0	1

Time, ¾ second.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.	24.
Mean record	958	994	1000	995	986	975	962	946	930	915	898	881
¾-second control	578	550	524	500	478	459	440	422	406	392	378	365
Solution*	47	42	39	38	34	32	30	27	24	22	20	—
Remainder	2	0	—1	—1	0	0	1	1	—1	0	0	—

* For absolute units, calories per gram per second, multiply by $0 \cdot 0902 \times 10^{-6}$.

practically the same solution, and they require respectively not more than 1/4 and 1/16 of the amount of calculation. With the apparatus available it is clear that no greater accuracy would be reached by working in time units less than 1 second. In this case the initial heat is practically the same at the

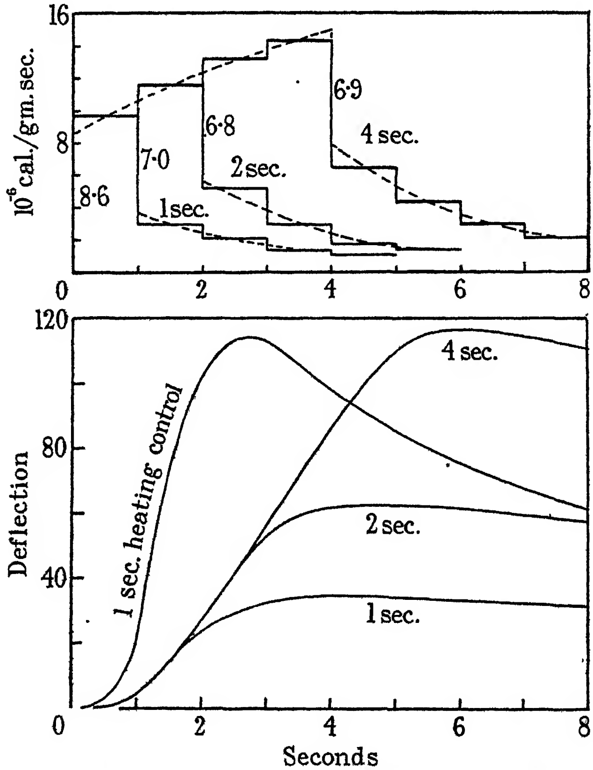

FIG. 2.—*Experiment of May* 31, 1932, at 19° C. Records begun about 4 hours after dissection. Maximal shocks, 370/second.

Below: Records reduced to same scale, 4 seconds stimuli (mean of 26), 2-seconds (mean of 24), 1-second (mean of 25), and 1-second heating control.

Above: Analysis of records for the three cases. The broken lines are smooth curves interpolated between the blocks. The initial heat at the beginning is $8 \cdot 6 \times 10^{-6}$ cal./gm. sec.; at the end of 1 second it has fallen to $7 \cdot 0 \times 10^{-6}$ cal./gm. sec., after which, for the next 3 seconds, it does not fall appreciably further.

end as at the beginning of stimulation; consequently the curve is of type I (or II), fig. 6.

Another analysis of 12 seconds stimulation with rapid recording is given elsewhere (Hill, 1932, *b*, fig. 7, p. 21). A curve properly interpolated between the solutions gives 7×10^{-6} cal./gm. sec. as the initial heat rate at the beginning

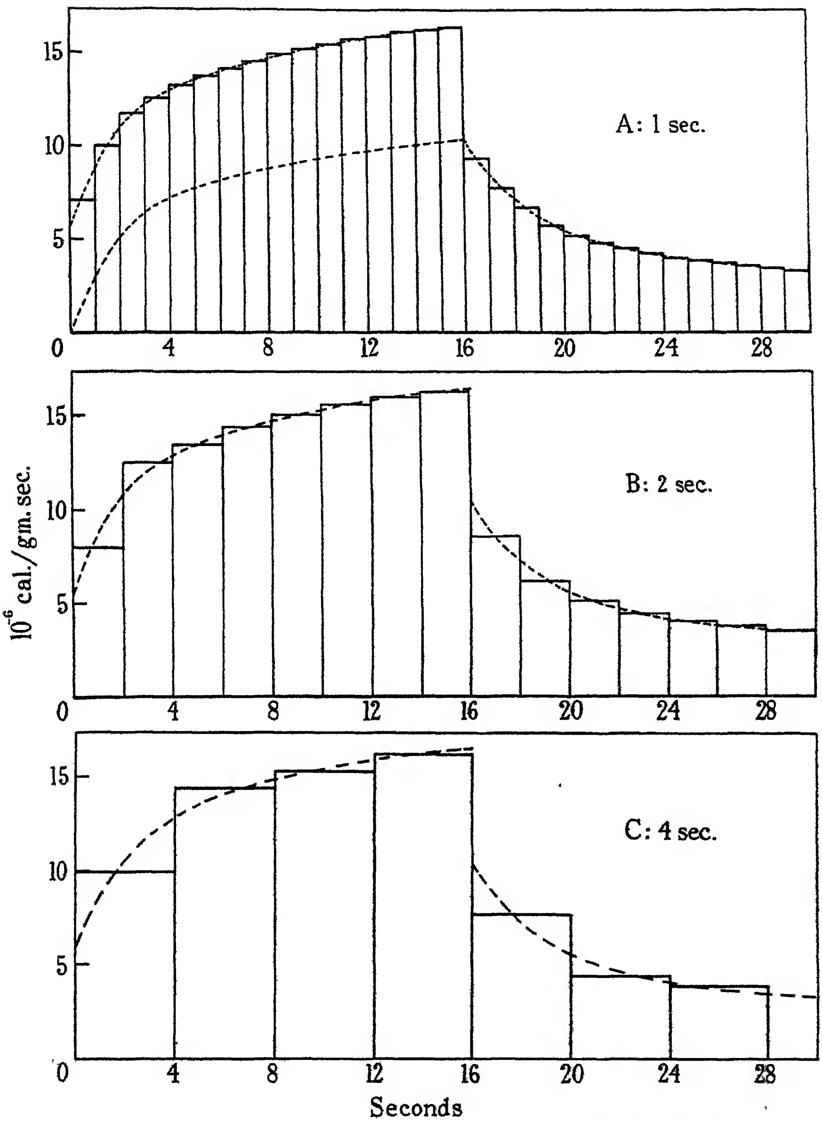

FIG. 3.—Analysis of records of 16 seconds stimulus, A in 1-second, B in 2-seconds, C in 4-seconds time units, for comparison. See Table II for description. The upper broken line is interpolated to give the course of the heat production. The lower broken line in A gives the recovery heat alone, after subtracting the initial heat from the total.

Table II.—*Experiment of October* 11, 1932, at 18·5° C., with four pairs of nerves of Hungarian frog, *R. esc.* 16-seconds stimuli at 8-minutes intervals, mean of 19 given in 0·05 mm. units. Nearly maximal stimulation, at 380/sec. for 14, 575/sec. for 2, 600/sec. for 3. 1-second heating control = 133×10^{-6} cal./gm.

Time, seconds:	0.	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.
Mean deflection	0	13	68	146	230	314	393	468	541	610	676	740	801	858	913	965
Control	0	247	956	1000	898	806	726	657	598	549	505	469	436	407	381	358
Solution A	53	75	88	94	99	103	106	109	112	114	116	118	119	121	122	123
Remainder	0	0	−1	0	0	1	1	0	0	0	0	0	0	0	−1	−2
Solution B	60	—	94	—	101	—	108	—	113	—	117	—	120	—	122	—
Remainder	0	—	−3	—	3	—	1	—	0	—	−1	—	0	—	0	—
Solution C	74	—	—	—	108	—	—	—	114	—	—	—	121	—	—	—
Remainder	0	—	—	—	0	—	—	—	0	—	—	—	0	—	—	—

Time, seconds:	16.	17.	18.	19.	20.	21.	22.	23.	24.	25.	26.	27.	28.	29.	30.
Mean deflection	1017	1055	1043	1018	989	958	927	897	870	843	819	797	776	757	739
Control	337	318	302	287	273	260	248	238	228	219	210	202	194	186	179
Solution A	70	58	50	43	39	36	34	32	30	29	28	27	26	25	—
Remainder	−1	2	−1	−2	−2	−1	2	−1	0	−1	−1	0	0	0	1
Solution B	64	—	46	—	38	—	33	—	30	—	28	—	26	—	—
Remainder	0	—	−3	—	2	—	1	—	0	—	−1	—	0	—	0
Solution C	58	—	—	—	33	—	—	—	29	—	—	—	—	—	—
Remainder	0	—	—	—	0	—	—	—	0	—	—	—	—	—	—

Solution A is in 1-second time units by the 1-second control; B is in 2-second units by a built-up 2-second control; C is in 4-second units by a built-up 4-second control. Each solution is in thousandths of the corresponding control. The initial heat rate is $5 \cdot 7 \times 10^{-6}$ cal./gm. sec. at the beginning and $6 \cdot 0 \times 10^{-6}$ cal./gm. sec. at the end of the stimulus. See fig. 3.

and 5×10^{-6} cal./gm. sec. at the end of stimulation. The curve is of type III, fig. 6.

These examples make it clear that there is an abrupt rise in the rate of heat production at the beginning of stimulation and an abrupt fall at its end. The only possible interpretation of these sudden changes is that during stimulation there is a distinct phase of heat production—what we call the initial heat—which starts and ends abruptly. The available technique does not enable us to decide whether the initial heat corresponding to a given impulse occurs actually during the impulse or a short time afterwards. It is arguable that none of the heat appears during the actual passage of the conducted wave but only, say, 1/5 of a second afterwards. However this may be, there is no doubt from the analyses that there is a separate phase of heat production closely associated, if not actually simultaneous with the conduction of the impulse.

In earlier experiments in which analysis was carried out in 1-second time units, the first block of the analysis was taken as representing the initial heat. This, it is clear, involves a certain error, for even the first second must contain recovery heat. When analysis is done in 2-seconds time units the error would be more serious. The intersection, therefore, with the vertical axis, of a smooth curve drawn through the final results of the analysis has been used to give the value of the initial heat rate at the beginning of stimulation. The intersection of a smooth curve drawn through the results after stimulation, with the ordinate at the end of stimulation, similarly supplies the initial heat rate at that moment. Very consistent estimates of the abrupt rise and fall of heat rate at the beginning and end of stimulation can be made in this way. These abrupt changes are what is meant by the initial heat. They are not necessarily the same at the beginning and end of a stimulus. Usually there is a slight decrease, sometimes a considerable decrease, in the initial heat rate during a stimulus (say) of 16 seconds duration at a high frequency.

Referring now to the recovery heat, it is evident from the curves given here and elsewhere that there is a rather rapid fall in rate immediately after the end of stimulation, which is followed by a very slow fall continuing, as will be shown in subsequent papers, for 25 to 35 minutes at 20° C. It is possible to reach a steady rate of heat production if stimulation, at not too high a frequency, be continued for 25 to 35 minutes. This agrees with the finding that the recovery heat production from a single stimulus persists for a similar time. Let us assume that this slow recovery process is represented by an exponential term Be^{-bt}, where b is given the value 0·003, which is such that the slow process is half complete in about 4 minutes, 99% complete in about 25 minutes.

From the analyses given it is evident that the rapid phase of recovery is complete in a few seconds, and measurement of the fall of the curve after the end of stimulation in a number of records has given $2\frac{1}{2}$ seconds as about the time required for the rapid process to reach half its initial rate. Let us assume that the rapid recovery phase is represented by another exponential term Ae^{-at}, where a is taken as 0·25, which is such that the "A" process would be half complete in $2\frac{1}{2}$ seconds, 99% complete in 18 seconds. Let I be the rate of initial heat production during a stimulus, and suppose that any element $I\delta\theta$ of initial heat is followed by a proportional element of recovery heat, whose rate at time t is

$$\frac{I\delta\theta}{k}(Ae^{-at}+Be^{-bt}).$$

The total amount of recovery heat, obtained by integration, is

$$\frac{I\delta\theta}{k}\left(\frac{A}{a}+\frac{B}{b}\right),$$

so the ratio of recovery to initial heat is

$$\frac{1}{k}\left(\frac{A}{a}+\frac{B}{b}\right).$$

Let us take

$$\frac{1}{k}\left(\frac{A}{a}+\frac{B}{b}\right)=24{\cdot}7,$$

which is about the value found in a subsequent paper.

Let stimulation continue for time T and then stop. At any subsequent time t the rate of recovery heat production is

$$\int_{\theta=0}^{\theta=T}\frac{1}{k}\left[Ae^{-a(t-\theta)}+Be^{-b(t-\theta)}\right]d\theta,$$

which (if I be constant) is

$$\frac{1}{k}\left[\frac{A}{a}\left(e^{-a(t-T)}-e^{-at}\right)+\frac{B}{b}\left(e^{-b(t-T)}-e^{-bt}\right)\right].$$

The rate of the "A" process at time $t=T$, *i.e.*, immediately at the end of stimulation, is $\frac{A}{a}(1-e^{-aT})$, that of the "B" process $\frac{B}{b}(1-e^{-bT})$. The former can be read off from the analyses, as the drop in recovery heat that occurs in 15 seconds or so. The latter is what then remains.

Thus, knowing a and b, A, B and k can be determined in a number of experiments. Round values so found are $A/a=10$, $B/b=200$, $k=8{\cdot}5$.

Fig. 4 shows the rate of heat production during and after stimuli of 2, 4, 8, 16 and 32 seconds duration, as calculated from the formulæ and constants given above, assuming a uniform rate of initial heat production of 6. The curves are very similar to those actually observed; see for example fig. 2 above, and figs. 11, 12 and 13 in a previous paper (Hill, 1932, *a*). The likeness is most striking in those cases in which the rate of initial heat production is approximately constant during stimulation. In many experiments, however, *e.g.*, that of fig. 1 above, the rate of initial heat production diminishes considerably during a stimulus; this is particularly so at 0° C., see, *e.g.*, figs. 14 and

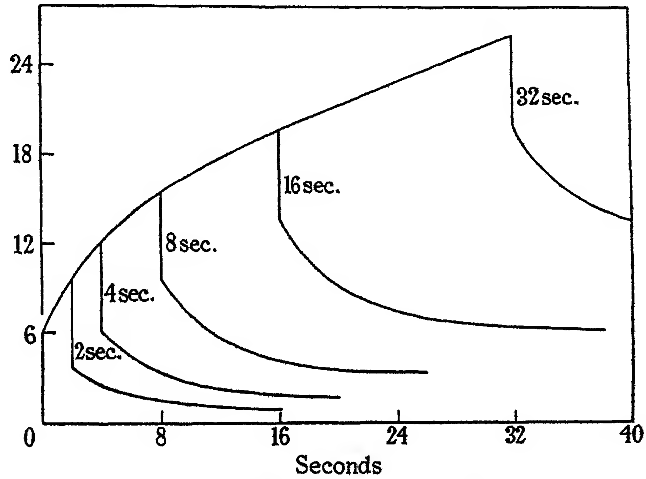

Fig. 4.—Calculated curves of total heat rate, during and after stimuli of various lengths, assuming a uniform rate of initial heat production of 6 during stimulation and the equations and constants given in the text.

16 of the paper referred to above. The consequence of this is that the rate of total heat production does not go on rising so fast as in the calculated curve of fig. 4; it may become constant, or even begin to diminish. Indeed, with a stimulus of sufficiently high frequency continued for some time such "fatigue" invariably occurs. It is possible to take account, in the equations, of the decrease of I, the initial heat rate, and in fig. 5 is an example of 32 seconds stimulation calculated from the same constants as before but assuming that the initial heat rate starting from 8·5 falls exponentially to 5·3 at the end of 32 seconds, and would fall further to 2·5 were stimulation continued indefinitely. This curve is so strikingly similar, and in such varied respects, to many actually observed that little doubt can remain of the general validity of the assumptions on which it is calculated.

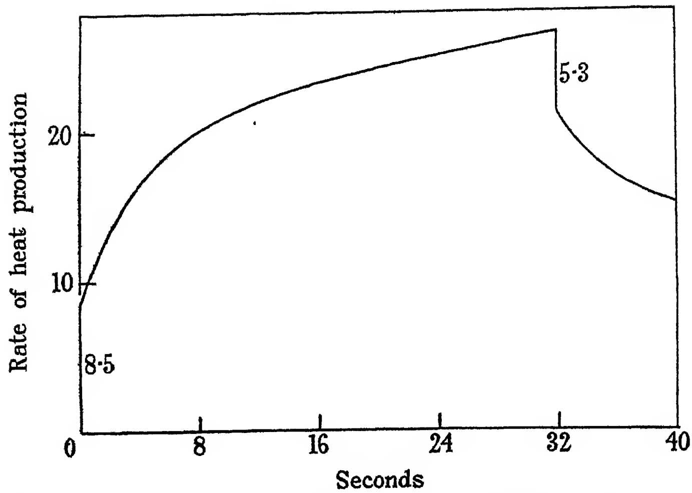

FIG. 5.—Calculated curve of total heat rate, during and after a 32 seconds stimulus, assuming equations and constants given in the text. The rate of initial heat production is supposed to start at 8·5 and to decrease exponentially to 5·3 at 32 seconds (to 2·55 at $t = \infty$).

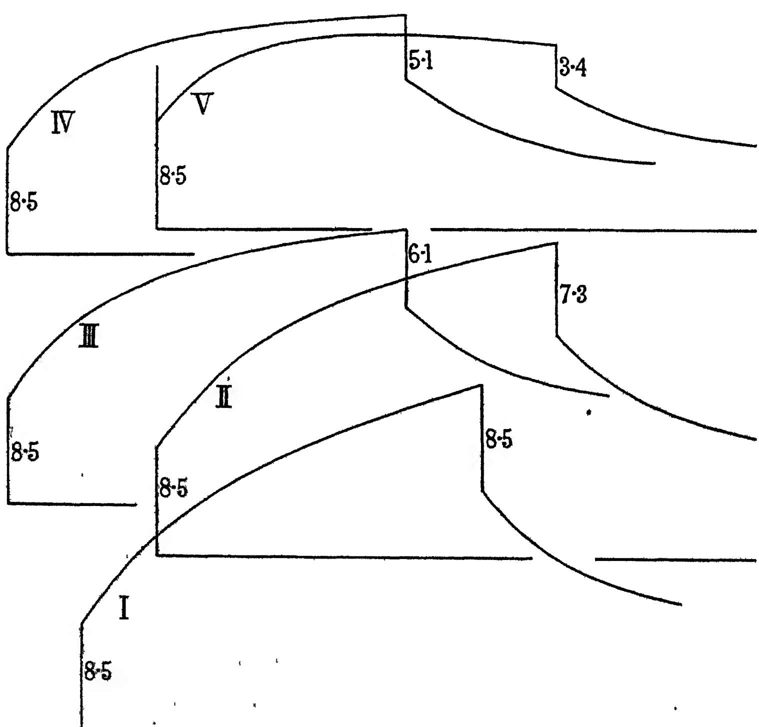

FIG. 6.—Calculated curves of total heat rate, during and after a 16 seconds stimulus, assuming the equations and constants given in the text. The rate of initial heat production is supposed to start always at the same value (8·5) and then either to continue uniformly (curve I), or to decrease exponentially (curves II to V) as follows: II—To 7·3 at 16 seconds, to 4·24 at ∞ ; III—To 6·15 at 16 seconds, to 4·25 at ∞ ; IV—To 5·1 at 16 seconds, to 4·25 at ∞ ; V—To 3·4 at 16 seconds, to 1·7 at ∞.

In fig. 6 various calculated curves are given for the experiment with 16 seconds stimulation, with the same constants as before, but with the initial heat decreasing to various degrees during the stimulus. No case has been found which does not conform to one or other of the types shown. It is clear that "fatigue" setting in during stimulation is the reason why the heat rate curves deviate from a constant type.

It is possible to change the form of the heat rate curve by various means, some of which will be referred to in the following papers. Fig. 7 gives an example of the same nerves, first in 5% CO_2, then later in 10% CO_2, in oxygen.

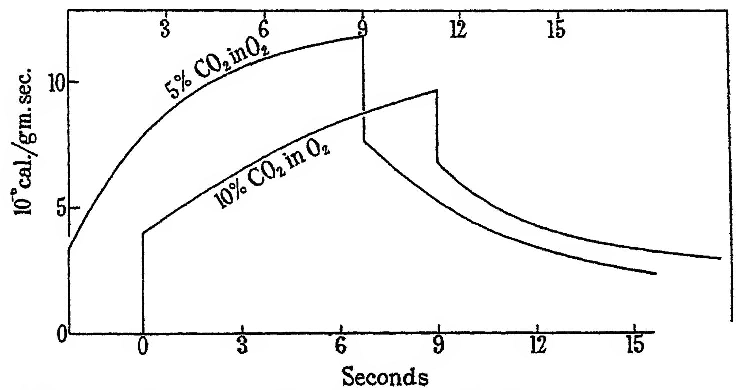

Fig. 7.—Effect of CO_2 on nerve heat production. Experiment of October 24, 1932, at 17½° C. Quick galvanometer system. Analyses in ¾-second time units of mean of: 5% CO_2, 14 records; 10% CO_2, 18 records; at 3 to 4 minute intervals, of 9 seconds stimulation by nearly maximal shocks, 420/second. Nerves dissected 10–11 a.m., 5% CO_2 in at 12.45 p.m., records 2–3 p.m., 10% CO_2 in at 3.15 p.m., records 4.15 to 5.15 p.m. Note the slower rise, due to decreasing initial heat rate, in 10% CO_2.

In the former the initial heat remained constant, or even increased a little, during stimulation; in the latter it decreased considerably. The CO_2 may have caused a "Treppe" at the lower concentration, and an increase of the refractory period during stimulation at the higher.

Summary.

(1) The process of activity in frog's medullated nerve occurs in at least three phases:—

(*a*) That in which the initial heat is liberated: this may or may not coincide in time with the actual transmission of the impulse, but it certainly does not lag behind it by more than a fraction of a second.

(b) That in which the rapid "A" process of recovery occurs: at 20° C. in frog's nerve this runs to half completion in 2 or 3 seconds and to completion (within 1%) in about 20 seconds.

(c) That in which the slow "B" process of recovery occurs: this runs to half completion in 4 or 5 minutes, to completion (within 1%) in 25 to 35 minutes.

(2) The total heat liberated in the rapid "A" process of recovery is about equal to the initial heat; that in the slow "B" process is many times greater.

(3) The initial heat rate may diminish rather rapidly during stimulation, particularly at a high frequency or a low temperature.

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The Steady State of Heat Production of Nerve.

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(Received May 2, 1933.)

[Plate 11.]

At rest at 20° C. in oxygen a frog's nerve produces heat at a rate of about 70×10^{-6} cal./gm. sec. (Beresina, 1932). All activity is superimposed upon this "basal" state. If a nerve be stimulated at not too high a frequency its rate of heat production rises, until in 25 to 40 minutes (at 20° C.) it reaches a steady level in excess of the "basal." If the stimulus be then stopped the rate of heat production gradually returns to its initial "basal" value. The process is illustrated in fig. 1. The frequency was low (9·7 shocks per second) and the final value reached by the extra heat production after 35 minutes stimulation was $9 \cdot 7 \times 10^{-6}$ cal./gm. sec. or 10^{-6} cal./gm. impulse.

* Tsing Hua University Fellow.

In fig. 1 galvanometer deflection is given without analysis: the latter would make the curve rise and fall rather more sharply at the beginning and end of stimulation. Fig. 2 gives such analyses, in 5-second units without remainders. Of these, (A) is of the beginning of stimulation, (B) of the end. The heat

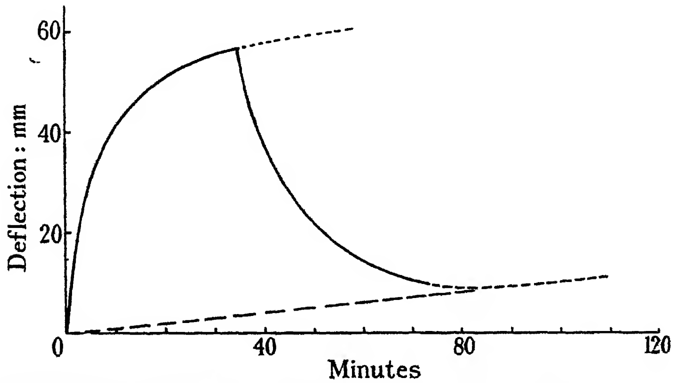

FIG. 1.—*Experiment of January* 27, 1933, 18·9° C. Heat production of nerve during steady state at 9·7 shocks per second. Condenser discharges, 6 v. 2·2 μF., half discharge time 1·3 σ. Stimulation lasted for 35 minutes, at the end of this time the rate of heat production was very nearly but not quite constant. Steady heat production = $9 \cdot 7 \times 10^{-6}$ cal./gm. sec. = 10^{-6} cal./gm. impulse. The creep of the zero was about 1 mm. in 10 minutes.

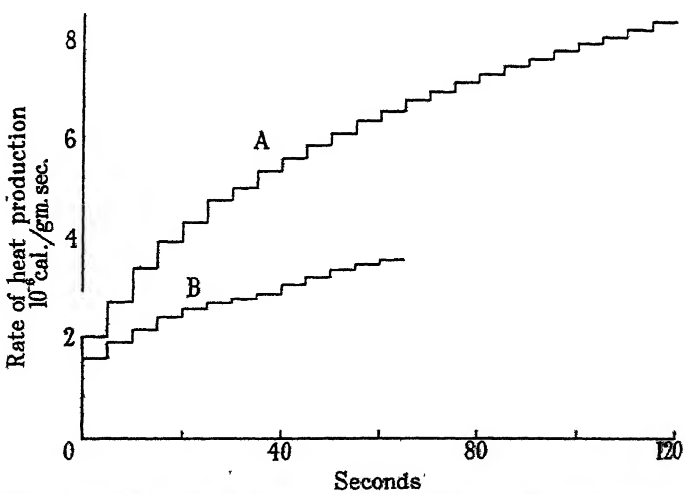

FIG. 2.—*Experiment of December* 1, 1932, 19·7° C. Analysis in 5-second units of rate of heat production at beginning and end of a long stimulus. (A) beginning of mean of six records at a mean frequency of stimulation of 13 per second. (B) end of mean of 3 records after a mean frequency of 10·4 per second. Note: (B) is negative, starting at the end of stimulation, and measured downwards from the base line representing the steady heat production during stimulation.

production in (B) is negative—just as (A) represents the sudden change upwards from the basal rate at the beginning of stimulation, so (B) represents the sudden change downwards at the end of stimulation from the steady level during stimulation.

During a steady state of continual stimulation at constant frequency, which is reached in 25 to 40 minutes at 20° C. (the same, as will be seen in a following paper, as the duration of the recovery process), the total heat production at any moment represents the sum of (*a*) the initial heat due to the stimulus at that moment and (*b*) the accumulation of the recovery heat from all the stimuli in the preceding 25 to 40 minutes. A steady state is possible only if the frequency of stimulation be not too high; otherwise fatigue sets in.

Method.

In all respects but four the method employed was the same as that described by Hill (1932).

(A) The falling plate camera was replaced by one in which a continuous record could be taken round a drum carrying a strip of photographic paper 1 metre long.

(B) A rapid recording system being unnecessary, galvanometer amplification was discarded and a single moving-coil Zernicke galvanometer (Zc) was used. This was given a rather smaller mirror, and was mounted on a Julius suspension 4½ metres from the camera. Its sensitivity and stability were such that 10^{-10} amp. gave a deflection of 1 mm. usually readable to 0·1 mm. on the photographic record. One-third of the full deflection was reached in 1 second.

(C) The thermopile was replaced by a similar one of about the same sensitivity, but fitted, fig. 3, Plate 11, with a large silver block through grooves in which the nerves ran from stimulating electrodes to thermopile. This prevented heat produced physically at the stimulating electrodes from being conducted to the thermopile, which is important in the measurement of heat over long periods. For greater stability the brass frame was made much heavier and an insulated screw-on brass cover was used.

(D) A new thermostat was constructed which has so improved the stability of the system as to give a different order of accuracy to the results. In fig. 1 the zero creep is only about 1 mm. in 10 minutes and reasonably constant at that: 1 mm. represents about 2 millionths of a degree. With previous arrangements extraneous disturbances might obscure the smaller rates of heat production to be observed.

The New Thermostat.—The principle of the new thermostat is the same as that described by Tian (1923). It uses alternate layers of high and low thermal conductivity in a room maintained by a regulator and electric heating at an approximately constant temperature. The inside chamber, fig. 3, (1) A, Plate 11, consists of a heavy copper cylinder 22 cm. deep, 11 cm. internal diameter, with a wall 2 cm. thick. It is mounted in, but insulated by bakelite stops, by kapok and by pitch from a brass cylinder (1) B, 38 cm. deep, 23 cm. internal diameter, with a wall 1·3 cm. thick. This cylinder is provided with a brass cover which can be screwed down to be completely airtight and is supplied with stop-cocks for gas inlet and outlet and for removing solutions in the nerve chamber. With the stop-cocks closed the thermopile is perfectly protected from changes of pressure occurring outside; this results, particularly on windy days, in a great improvement in the stability of the readings.

The brass cylinder is mounted on insulating stops in a wooden box (1) C, 60 cm. square and 80 cm. high. It is insulated from the box by kapok and covered with a thick cushion of kapok before the cover of the box is put on. The inside of the box is lined with sheet copper. The inner copper cylinder is filled with thick ("medicinal") paraffin oil so that between the brass wall of the nerve chamber and the air of the room are the following alternate non-conducting and conducting layers: oil, copper, kapok, brass, kapok, copper sheet, wood.

By virtue of the great heat capacity and the high thermal conductivity of its brass and copper cylinders, together with its thick layers of excellent heat insulators, the thermostat ensures great uniformity of temperature and enormous lag even to violent fluctuations of room temperature. In order, however, to work under the best conditions the room temperature was always regulated within 0·5° C. for one or two days before an experiment, in order to bring the temperature inside the thermostat approximately to that of the room. At the beginning of an experiment the temperature inside the thermostat was measured and the room regulated at a slightly higher temperature so that any gradual change of temperature* in the thermostat during an experiment should be in the warming direction. This avoids the possibility of supersaturation occurring at intervals in the nerve chamber, which may easily result in violent fluctuations owing to the deposition of moisture on the nerve (see Hill, 1932, p. 119).

* For this type of work absolute constancy of temperature is not needed, but only that the rate of change should be slow, constant, and continuous for long periods without fluctuations. A new thermostat, recently completed, is placed inside a large box, within which—instead of in the room—the temperature is kept constant by a regulator.

At the beginning of an experiment the nerves on the thermopile, in their chamber filled with Ringer's solution, are placed in the oil bath in the copper cylinder. The thermostat is closed and covered. The solution in the chamber is stirred by a stream of oxygen and the oil bath by a stream of air. After about 2 hours, to ensure temperature equilibration and approximate equilibrium of vapour pressure between nerve and solution, the Ringer's solution is withdrawn and replaced by oxygen, and the stopcocks are closed by pulling on strings suitably attached to them. In about 1 hour the experiment can be started.

Sensitivity.—Calibration was carried out in each experiment by a series of condenser discharges of known voltage and capacity. With the same thermopile and galvanometer the sensitivity is nearly constant; the following numbers give the value of 1 mm. of steady deflection, in calories per second, each representing a separate experiment: $2{\cdot}35 \times 10^{-8}$, $2{\cdot}3 \times 10^{-8}$, $2{\cdot}3 \times 10^{-8}$, $2{\cdot}5 \times 10^{-8}$, $2{\cdot}3 \times 10^{-8}$, $2{\cdot}95 \times 10^{-8}$, $3{\cdot}1 \times 10^{-8}$, $2{\cdot}6 \times 10^{-8}$.

With nerves weighing about 150 mg., an average value of 1 mm. of steady deflection is about 16×10^{-8} calorie per gram of nerve per second.

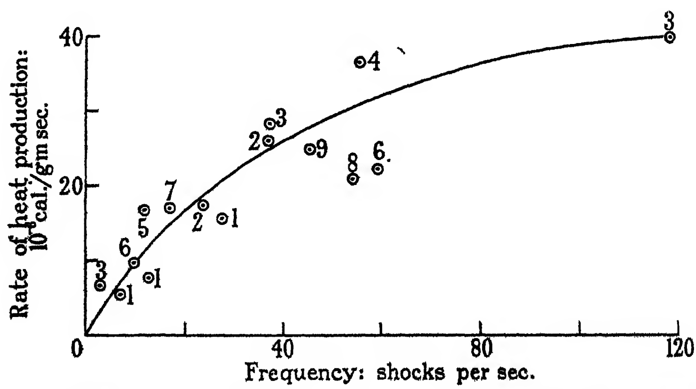

FIG. 4.—Rate of total heat production of frogs' nerves at about 19° C. (in excess of "basal") during steady stimulation at various frequencies. The scatter is due to the fact that the observations were made in 9 separate experiments over a period of two months. The numbers represent the separate experiments of Table I.

Results.

The results of nine different experiments at 18 frequencies are given in Table I and fig. 4. One fact is at once obvious, that whereas at a low frequency of stimulation the rate of heat production only gradually reaches its maximum, at which it then remains constant, at a high frequency on the contrary it

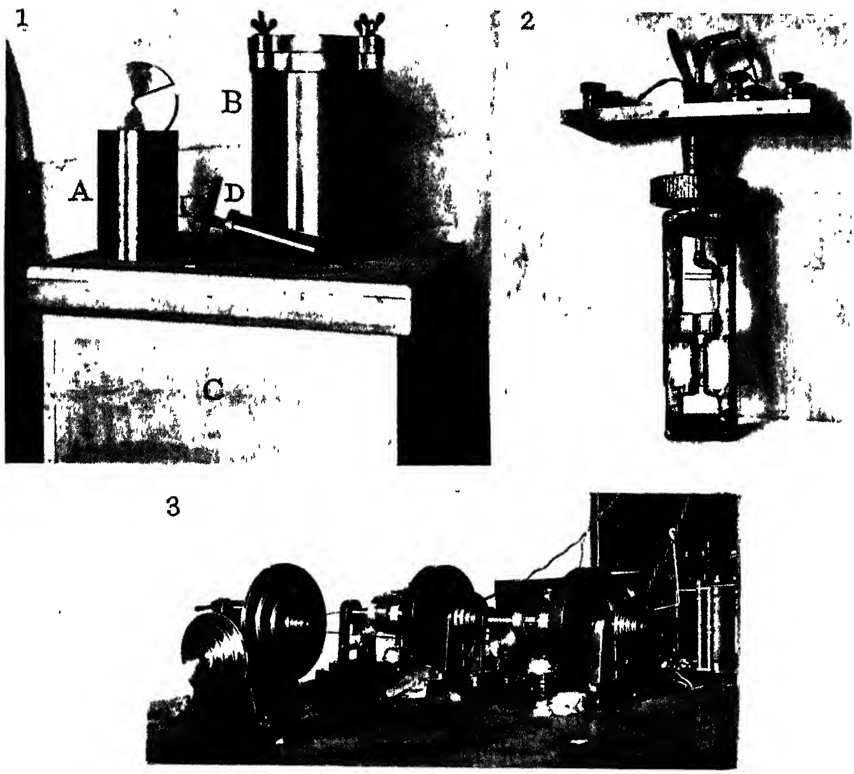

Fig. 3.—*Apparatus.* 1. Thermostat, component parts before erection. A, copper cylinder with cover ; B, brass cylinder with screw-on cover, to hold copper cylinder ; C, wooden box lined with copper sheet with cover. to hold brass cylinder ; D, nerve thermopile chamber.

2. Nerve thermopile, with cover removed, showing stimulating electrodes above, grooved silver block for cutting off heat leak in the middle, groove containing hot junctions of thermopile plate below. The thermopile has 145 couples of constantan-iron and a resistance of 66 ohms.

3. Commutators used for stimulating by condenser charges and discharges. One commutator is shown connected to a pulley and motor. The two commutators cna be driven independently, and by pressing a Morse key a stimulus supplied by a given battery and condenser can be instantly switched from the first to the second commutator and so its frequency altered.

Table I.—Rate of total heat production by frogs' nerves during a steady state of stimulation.

Date:	December 15.			January 3.										
Temperature °C.:	20·1			18·8										
Frequency, shocks per second:	27·5	12·8	7·04	36·4							23·5			
Duration of stimulus, min.	51	25	24	31	36	41	46	51	56	61	22	27	32	37
Cal./gm. per sec. × 10^{-6}	15·7	7·6	5·3	25·8	25·8	25·8	25·5	25·1	24·8	24·4	16·9	17·1	17·4	17·4
Cal./gm. per impulse × 10^{-7}	5·7	5·9	7·6	7·1	7·1	7·1	7·0	6·9	6·8	6·7	7·2	7·3	7·4	7·4

Date:	January 3. (*contd.*)		January 5.											
Temperature °C.:	18·8		19·2											
Frequency, shocks per second:	23·5		3	11·6	37				118					
Duration of stimulus, min.	42	47	25	41	32	37	42	47	15	20	25	30	35	40
Cal./gm. per sec. × 10^{-6}	16·9	16·9	6·6	16·8	28·5	27·8	27·4	26·6	40	38	37	35	34	33
Cal./gm. per impulse × 10^{-7}	7·2	7·2	22	14·5	7·7	7·5	7·4	7·2	3·4	3·2	3·1	3·0	2·9	2·8

Table I—(*continued*).

Date:	January 5. (*contd.*)		January 11.					Jan. 13	January 27.					
Temperature °C.:	19·2		18·9					18·7	18·9					
Frequency, shocks per second:	118		55	700				10·9	9·7	59				
Duration of stimulus, min.	45	50	20–40	15	20	25	30	40	35	22	27	32	37	42
Cal./gm. per sec. × 10^{-6}	32	31	36·6	37·4	34·8	32·2	30·0	15	9·7	22·3	22·5	22·3	21·9	21·7
Cal./gm. per impulse × 10^{-7}	2·7	2·6	6·7	0·53	0·50	0·46	0·43	13·8	10	3·8	3·8	3·8	3·7	3·7

Date:	January 27. (*contd.*)							Feb. 6	February 15.		Feb. 21
Temperature °C.:	18·9							19·2	18·6		18·2
Frequency, shocks per second:	59	183						17	54		45
Duration of stimulus, min.	47	22	27	32	37	42	47	20	23	30	30
Cal./gm. per sec. × 10^{-6}	21·1	31·9	30·6	29·8	28·4	27·0	26·2	17	21	19·7	25
Cal./gm. per impulse × 10^{-7}	3·6	1·74	1·67	1·63	1·55	1·47	1·43	10	3·9	3·6	5·5

Stimulation by maximal condenser discharges. In most cases the half-discharge time was about 0·18 σ. In the experiments of January 13 and January 27 large capacities were used, the half-discharge times being respectively 2·3 σ and 1·3 σ; in the latter experiment, however, the high frequency stimulus (183/sec.) had a half-discharge time of 0·024 σ. See fig. 4.

reaches its maximum rather quickly and then falls off. At a low frequency a genuinely steady state is attained; at a high one a steady state is apparently impossible.

All the stages in the transition from low to high frequency can be seen. For example, in fig. 1 the steady condition at 9·7 shocks/sec. had not been quite reached even in 35 minutes. In the experiment of January 3 at 36·4 shocks/sec. the heat production remained at its maximum till the 41st minute, after which it began slowly to fall; similar effects are seen at 23·5 shocks/sec. on the same day, at 37 shocks/sec. on January 5 and at 59 shocks/sec. on January 27. At 118 shocks/sec. on January 5 the maximum rate was reached within 15 minutes; at 183 shocks/sec. on January 27 within 22 minutes; at 700 shocks/sec. on January 11 within 15 minutes; in all these experiments the maximum was followed by a steady fall.

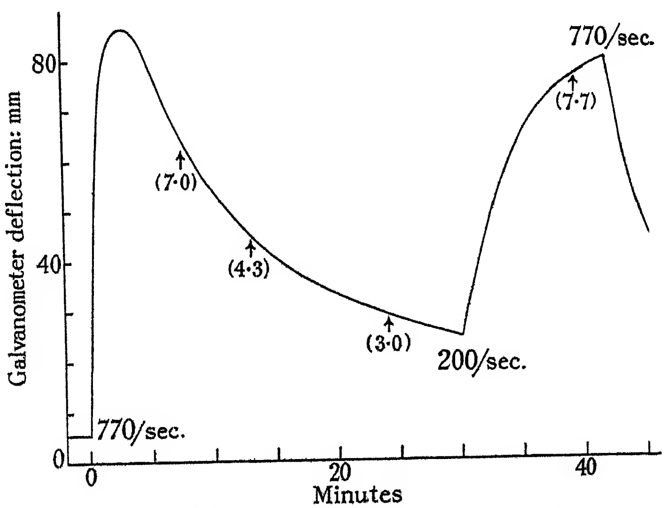

FIG. 5.—*Experiment of February* 15, 1933, 18·6° C. To show rapid onset of "fatigue" during stimulation of nerve at high frequency (770/sec.) but quick "recovery" on changing to a lower frequency (200/sec.). Note that a return to the high frequency caused an immediate diminution in the response. Abscissa, time: ordinate, galvanometer deflection recording rate of heat production. In a steady state 1 mm. deflection represents $15 \cdot 8 \times 10^{-8}$ cal./gm. sec. Stimulus, condenser discharges 14 v. 0·2 μF., half discharge time 0·12 σ. The numbers in brackets represent the negative response in millimetres due to omitting the stimulus for 16 seconds.

A striking example of the "fatigue" effect is seen in fig. 5 where, at 770 shocks/sec., the rate of heat production reached its maximum in less than 3 minutes and then fell rapidly. At 30 minutes the frequency was lowered to 200/sec. and the rate of heat production began to increase at once. After

14 minutes of this the frequency was raised again to 770 shocks/sec., and the rate of heat production immediately began to fall again. The condenser discharges used for stimulation were half complete in $0 \cdot 12\,\sigma$, so no overlapping of these can have occurred.

In fig. 4 the results of the various experiments are plotted together for comparison. Owing to the fact that these were obtained on different nerves over a period of more than 2 months there is a considerable degree of "scatter." It is clear nevertheless that the greatest rate of total heat production (in excess of "basal") is not more than about 40×10^{-6} cal./gm. sec., and is reached already at a frequency of about 100 shocks/sec.

At a low frequency the total heat *per impulse* is usually of the order of 10^{-6} cal./gm., though occasionally (*e.g.*, in the experiments of January 5 and 13) it may be somewhat greater. At a high frequency the heat per impulse is considerably less than 10^{-6} cal./gm.

According to Meyerhof and Schmitt (1929), working with nerves of *R. esc.* (winter), the extra oxygen consumption due to steady stimulation with 100 make and break shocks per second averages about 13 c.mm./gm. hr. According to Gerard (1927), working with the same nerves at $14 \cdot 6°$ C., steady stimulation at 280 make and break shocks per second causes an average extra oxygen consumption of 18 c.mm./gm. hr. Assuming that 1 c.c. of $O_2 \equiv 5$ cal. these correspond respectively to $18 \cdot 8 \times 10^{-6}$ and 25×10^{-6} cal./gm. sec.; assuming a temperature coefficient of $3 \cdot 0$ for 10° C., they would be 33×10^{-6} and 43×10^{-6} respectively at 20° C., and so in good agreement with the 40×10^{-6} cal./gm. sec. here found on similar nerves. In the present experiments the heat is due to impulses started by nearly optimal shocks and transmitted by physiological conduction several centimetres along the fibres before the thermopile is reached; the agreement confirms Gerard's objection (1932, p. 523) to Winterstein's contention that the extra oxygen consumption due to stimulation is caused directly by the applied current in the region of nerve on, or adjacent to, the stimulating electrodes.

We are indebted to Dr. F. J. W. Roughton for referring us to the thermostat described by Tian.

Summary.

If a frog's medullated nerve at 20° C. be subjected to continued stimulation at low or moderate frequency (up to 50 shocks per second) a steady state is reached in 25 to 40 minutes at which recovery keeps pace with breakdown and

the rate of heat production becomes constant. When the stimulus is discontinued the rate of heat production returns, also in 25 to 40 minutes, to its " basal " level.

At low frequencies the total heat per impulse is of the order of 10^{-6} calorie per gram.

At higher frequencies a steady state is impossible; the rate of heat production reaches a maximum and then declines. During the state of " fatigue " induced by high frequency stimulation a sudden change to a lower frequency may cause an immediate increase in the rate of heat production.

The greatest rate of heat production due to steady stimulation is of the order of 40×10^{-6} calorie per gram per second. This agrees with measurements made by others of the rate of oxygen consumption during continual stimulation.

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612 . 822 . 4

The Effect of Frequency of Stimulation on the Heat Production of Frog's Nerve.

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(Received May 2, 1933.)

The effect of frequency of stimulation on the heat production of nerve has been discussed already in several places, (*a*) by Gerard, Hill, and Zotterman (1927), (*b*) by Hill (1932), both for frog's medullated nerve, and (*c*) by Beresina and Feng (1932) for crab's non-medullated nerve. The matter is not so simple as has appeared hitherto: the heat per second, or per impulse, depends not only on the frequency but also on the duration of the stimulus. Referring, for example, to fig. 4 in the preceding paper, it is evident that the relation there shown between the rate of heat production during steady stimulation and the number of shocks per second is quite different from that given last year (Hill, 1932, fig. 5, p. 127) for a short stimulus. In the former, half the maximum rate is reached at 26 shocks/sec., in the latter at 80 shocks/sec.; the frequency scales, therefore, are in the ratio of about 1 to 3.

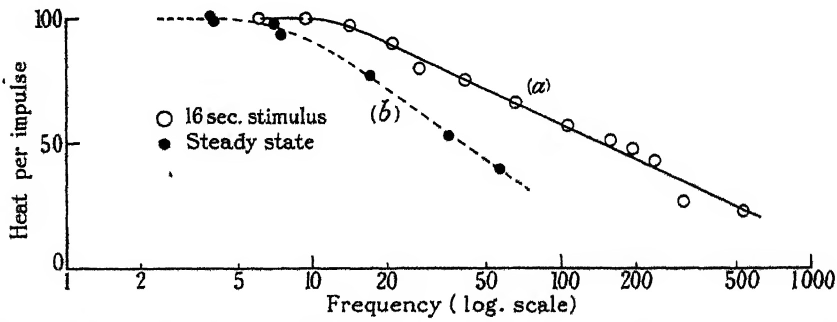

Fig. 1.—*Experiment of February* 24, 1933, about 19° C. Effect of frequency on the heat produced per impulse: (*a*) hollow circles, in a 16-second stimulus (mean of readings at 4, 8, 12, and 16 seconds); (*b*) full circles, in a steady state. The heat per impulse is taken as 100 at a low frequency in either case. The frequency is given on a logarithmic scale.

Fig. 1 shows the results of an experiment in which the effect of frequency on the heat per impulse was determined (*a*) for a 16-sec. stimulus and (*b*) for con-

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tinual stimulation until a steady state was reached. In order to keep the figure within reasonable dimensions the frequency is given on a logarithmic scale. In (*a*) the heat per impulse has fallen to one-half at a frequency of 140/sec., in (*b*) at a frequency of 40/sec.; the frequency scales again are in the ratio of about 1 to 3. In respect at least of those properties which result in heat production the time scale of nerve is diminished about three times by steady stimulation.

With a stimulus of short duration, the total heat rises continually with increasing frequency, the heat per impulse continually falls. At a low frequency (up to about 10/sec.), the heat per impulse, or per second, remains constant throughout the stimulus. At a high frequency, however, it decreases rather considerably as the stimulus is prolonged. If we give (*e.g.*) a 60-second stimulus, we can take as a measure of the heat, for comparison of different frequencies, the deflection at any fixed time, *e.g.*, 10 or 60 seconds. If the heat per impulse, or per second, were constant, and if the related rate of rise of the recovery process were independent of frequency, it would make no difference which time we chose. Actually it makes a considerable difference. An increase of frequency has a greater effect at short times than at long. Indeed, as we have seen (fig. 5 in the preceding paper), a stimulus of higher frequency continued long enough may give a smaller and not a greater response.

These effects can be seen in the experiments recorded below. The records were read at 10- or 8-second intervals and the deflections reduced to a common scale by assuming, what appears to be a fact, that at low frequencies the heat per impulse is the same at all durations. This gives a single factor for each duration by which the heat per impulse at any frequency can be expressed as a percentage of that at the lowest frequency.* It is evident that at the higher frequencies (and these are still rather low) the heat per impulse is a smaller fraction of that at the lowest frequency for long durations than for short. The relation, therefore, between heat and frequency is largely affected by the duration of the stimulus in which the heat is measured.

The numbers in square brackets are doubtful because of the smallness of the quantities observed.

Summary.

With nearly "optimal" condenser discharges for excitation the relation in nerve between heat production and frequency of stimulation is not simple. In general it is of the type described in previous papers, but it depends also

* Actually, since the deflections at the lowest frequency were very small, it is expressed as a percentage of a smoothed value at the lowest frequency.

Experiment of January 3, 1933. 18·8° C. 1-minute stimuli. Half-discharge time of shocks = 0·18 σ.

Frequency, shocks per second.	Heat per impulse, if measured by the deflection at the time noted.					
	10 secs.	20 secs.	30 secs.	40 secs.	50 secs.	60 secs.
1·22	[108]	97	93	98	102	102
1·7	[100]	94	95	91	92	96
2·9	86	87	85	84	86	86
5·9	84	85	87	88	87	87
8·2	76	79	79	78	77	77
17·1	71	69	69	66	63	61
36	63	59	55	53	49	47
78	47	41	38	36	34	32

Experiment of January 5, 1933. 19·25° C. 64-second stimuli every 6 or 7 minutes. Half-discharge time of shocks = 0·18 σ.

Frequency, shocks per second.	Heat per impulse, if measured by the deflection at the time noted.							
	8 secs.	16 secs.	24 secs.	32 secs.	40 secs.	48 secs.	56 secs.	64 secs.
1·28	—	98	90	100	103	100	97	98
2·31	101	102	102	99	106	104	103	102
3·79	93	98	93	101	99	98	100	104
7·94	94	90	92	91	90	90	89	88
12·1	96	93	93	89	86	85	83	80
20·8	78	80	82	78	73	71	69	66
35·3	89	77	71	67	59	56	54	50
61·6	63	55	51	47	42	40	39	37
133½	40	34	32	30	—	—	—	—
270	23	21	20	18	—	—	—	—

upon the duration of the stimulus. In a short stimulus a given increase of frequency has a greater relative effect on the heat than in a long stimulus. A nerve behaves as though it were slowed in its time relations by activity. During a steady state of continual stimulation the scale of frequency is altered about threefold. Within 1 minute of beginning stimulation at the higher frequencies the heat per impulse (or per second) has fallen considerably (*e.g.*, to one-half).

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612 . 822 . 4

The Relation between Initial and Recovery Heat Production in Frog's Nerve.

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The latest and best determination of the quantitative relation of the two chief phases of heat production in frog's nerve is that of Bronk (1931) who found the average ratio of total to initial heat to be 11. More recently Hill (1932) has given reason to believe that even Bronk's ratio is probably too low, and in the same paper (p. 148) has suggested an alternative method of estimating it based on the principle of the steady state. The present work arose from this suggestion and has led to the discovery of the profound effect of steady activity on the " efficiency " of the recovery process.

The steady state of nerve activity during continual stimulation has been discussed in a preceding paper. Its nature is such that the rate of recovery heat production at any moment corresponds to the rate of initial heat production at that moment. The total heat rate can easily be measured. The initial heat rate can be determined by omitting the stimulus for a short interval, when an analysis of the resulting deflection allows an estimate of the heat which immediately drops out as a result of omitting the stimulus, *i.e.*, of the initial heat.

I. *Preliminary Results.*

In a preliminary series of experiments carried out by one of us (T. P. F.) in collaboration with Mrs. M. Beresina during the early summer of 1932, the apparatus employed was identical with that described by Hill (1932). Only the deflections at the beginning and end of a prolonged stimulus and those due to brief omissions of stimulus (" gaps ") were photographed. The course of the heat production caused by the continuous stimulation was followed by reading every one minute the setting of a potential divider which balanced the e.m.f. due to the heat and kept the spot of light at some convenient point. Heating controls of one second duration were taken for the analysis and the potential divider was calibrated at the end of the experiment by heating the asphyxiated inexcitable nerves until a constant deflection was reached.

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With the thermopile used in these preliminary experiments (but not with that employed in the later ones) a correction for heat leak was necessary. This was made as described by Hill (1932, p. 133). Its magnitude, for continuous stimulation at about 50 shocks/sec., was generally about 3% of the observed physiological heat production.

During the course of a prolonged stimulus omissions of stimulation, or "gaps," usually of 8 seconds duration, were interposed at intervals of 6 minutes or so. The size of the negative deflection caused by such a gap is a measure of the effect of stimulation at the corresponding moment. If the effect of stimulation were constant throughout, the gaps would have a constant size. Actually this was never so. Shortly after the beginning of stimulation the gaps increased somewhat, then reached a maximum, then, if the frequency of stimulation was high, began to decrease again. At a low enough frequency the gaps showed only a slight decrease, or remained practically constant throughout, thus further indicating the existence of a genuine steady state.

The records of the gaps can be analysed in precisely the same way as the records of ordinary positive stimuli. The abrupt decrease in the rate of heat production at the beginning of a gap can be taken as the initial heat at that moment. The rate of total heat production can be measured by the deflection from the produced base line. The ratio of total to initial heat thus obtained in the preliminary experiments is given in Table I. Of these the first four were on normal nerves (Hungarian *R. esc.*, as in all subsequent experiments); in experiment 5 the medium round the nerves was not oxygen but oxygen containing 5% CO_2; in experiment 6 the nerve had been previously soaked for several hours in Ringer's solution of twice the normal strength in all constituents; in experiment 7, in Ringer's solution of half the normal strength.

Table I.—Ratio of total to initial heat for "gaps" in steady stimulation.

Experiment.	Condition.	Initial heat, 10^{-6} cal./gm. sec.	Total heat, 10^{-6} cal./gm. sec.	Total/Initial.
1	Normal, 1st day	3·3	29·2	9
	,, 2nd day	2·7	22·4	8·3
2	,, 1st stimulation	3·1	33	11
	,, 2nd stimulation	2·9	29·5	10
3	,,	2·9	29·3	10
4	,,	3·3	33·0	10
5	5% CO_2	2·9	29·0	10
6	2 Ringer	9·0	51·0	5·7
7	½ Ringer	1·8	31·0	17·0

About 20° C. 50 to 60 shocks/sec.

In normal nerve, Table I shows that the ratio of total to initial heat is about 10; 5% CO_2 has no effect; hypertonic (2R) and hypotonic ($\frac{1}{2}$R) Ringer's solution apparently exert a pronounced influence, the former decreasing, the latter increasing the ratio.

II. *The Ratio of Total to Initial Heat is widely different when Determined by different Methods.*

These preliminary experiments were interrupted by other work. In the meantime the new thermostat described in a preceding paper had been perfected, the Zernicke (Zc) galvanometer improved by replacing its mirror with a smaller one and by increasing its sensitivity somewhat, and the falling plate camera changed for one in which a continuous record could be taken round a drum. The preliminary experiments had given the ratio of total to initial heat as about 10. This, while agreeing closely with Bronk's result, appeared to be rather small in view of Hill's subsequent work on the initial heat. Advantage, therefore, was taken of the new arrangements to make measurements of the ratio of total to initial heat under various conditions, both by the method employed by Bronk and other previous observers, and also by that used in the preliminary experiments. Further experiments were made of high frequency stimuli superimposed, not upon a resting condition but upon one of slow steady stimulation. Unexpectedly, very different results were obtained under these various conditions.

For determining the ratio of total heat to initial heat by the usual method, *i.e.*, estimating (*a*) the initial heat by analysis of the abrupt rise and fall of heat rate at the beginning and end of stimulation, and (*b*) the total heat by means of the total deflection-time area, 24 seconds or 32 seconds stimuli at nearly maximal frequency were given to a completely resting nerve. It was found that the recovery heat from such stimuli at about 20° requires rather more than 30 minutes for its completion (see fig. 1). Photographic records were made of the base line for a minute or two before, and of the deflection for 10 minutes or so after, the stimulus. The camera was then closed, but opened again every minute to make a mark on the paper for 30 minutes or longer. Heating controls of 2 seconds duration were taken, the whole curve being recorded photographically. The analysis of the initial heat was carried out in 2 seconds time units, experience having shown that analysis in shorter time units gave no additional information, but required much greater labour in calculation. Analysis in these longer intervals made it practicable to apply

such relatively long stimuli as 24 and 32 seconds, accuracy being gained thereby in the estimation of the total heat.

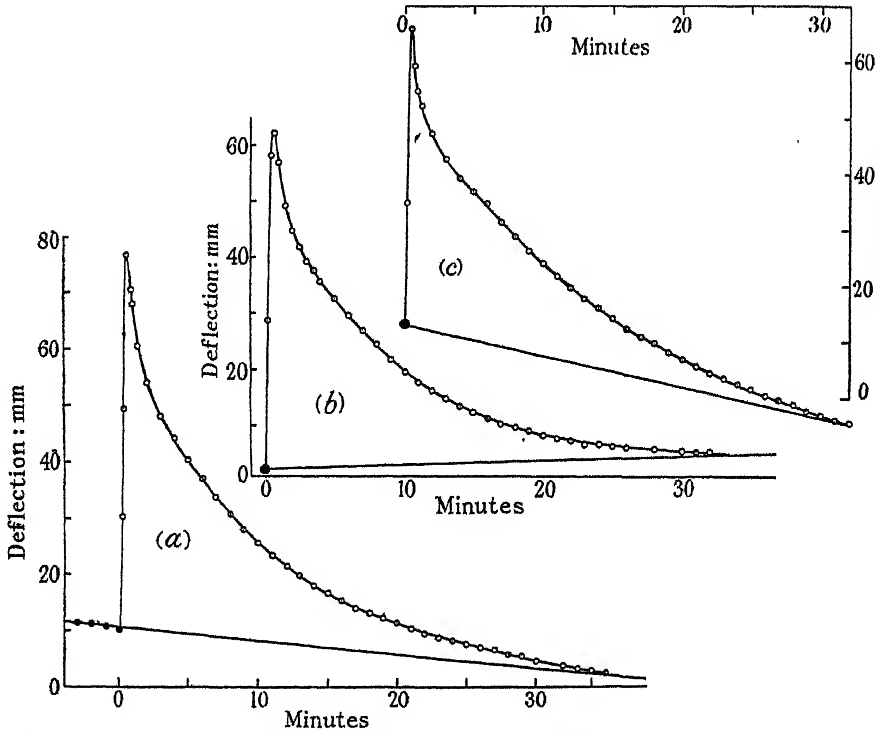

Fig. 1.—Galvanometer deflection (millimetres) recording the total heat production during and after a short stimulus to a previously resting nerve. The total heat is given by the area of the curve above the interpolated base line. (*a*) February 15 at 18·6° C., 32 seconds stimulus. (*b*) January 5 at 19·2° C., 32 seconds stimulus. (*c*) February 6 at 19·2° C., 24 seconds stimulus. Note that recovery requires 30 to 35 minutes for completion.

The Analysis.

It was found, nevertheless, that analysis even in 2 seconds units up to 32 or 40 seconds was too laborious by the usual method. A great saving of effort and time, and an improvement in accuracy, were achieved with a calculating machine. To take an example, the record of a 32 seconds stimulus was measured by 2 seconds intervals up to 48 seconds. It is first analysed in 4 seconds time units by means of a built up 4-seconds heating control. This process is practically mechanical, zero remainders being left except sometimes for the first block of all. The results of this analysis, which takes only a few minutes, are plotted on millimetre paper and a smooth curve is drawn through the tops of successive blocks. This curve gives interpolated solutions in 2 seconds time units, which—with a little experience—are generally very nearly right. To check their correctness they are used with the 2 seconds heating control to build up the record of a 32-seconds stimulus. The calculation is easily done with an adding and multiplying machine. Since the solutions are not quite

correct the calculated record differs slightly from the observed record. The differences are analysed by the 2-seconds heating control and the results applied as small corrections to the interpolated solutions. All arithmetical error is eliminated by the use of the calculating machine, so that even at the end of a long analysis accurate results are obtained, which is not possible by a slide rule with which errors gradually accumulate. Thus, the sudden decrease in the rate of heat production at the end of stimulation can be obtained by analysis with the same accuracy as the sudden increase at the beginning of stimulation. This is important for the accurate measurement of the whole initial heat during a long stimulus.

The initial heat rate was measured as described in the first paper of this series. The whole initial heat during a period of stimulation is assumed to be given by multiplying the average of the initial heat rates at the beginning and end of stimulation by the duration of the stimulus. This whole initial heat is the quantity required in calculating the ratio of total to initial heat. There is no need here to give examples of the analysis ; several have been given elsewhere, *e.g.*, in the first paper of the series. In the experiments of which the curves in fig. 1 represent the total heats the whole initial heats were (*a*) 203×10^{-6} m., cal./gm., (*b*) 138×10^{-6} cal./gm., and (*c*) 147×10^{-6} cal./gm. The total heat is given by the area of the curve above the interpolated base line. In these three experiments the total heats were : (*a*) 5640, (*b*) 4230, and (*c*) 3800 microcalories per gram respectively. The ratios, therefore, of total to initial heat were (*a*) 28, (*b*) 31, and (*c*) 26. Determinations of the same ratio in other sets of normal nerves, which are summarized in Table II, gave an average value of the ratio of total to initial heat of 29. The chief reason why this is so much greater than all previous determinations is that recovery has been followed so much longer ; it is possible that future investigation will show that we, too, have not followed it far enough.

Let us turn now to the results of the method used in the preliminary experiments, employing " gaps " in the stimulation during a steady state. Fig. 2 shows for comparison (A) the analysis of the heat due to an ordinary stimulus of 16 seconds at 54·7 shocks/sec., and (B) the analysis of the heat omitted, as the result of omitting the stimulus for 16 seconds, during a steady state of stimulation at 54·7 shocks/sec. (A) was made on the same set of nerves as (B) about an hour earlier. The records of gaps are very similar to those of ordinary stimuli (though, of course, in the opposite direction), the only obvious difference being that they return to their base line more quickly. Correspondingly in fig. 2 the analysis of the " gap " is very similar to that of the stimulus except that the recovery heat falls off more rapidly. The initial heat in the " gap " is just about the same as in the stimulus, viz., $4 \cdot 05 \times 10^{-6}$ cal./gm. sec. (This

Table II.—Initial and recovery heat: stimuli to resting nerves: Hungarian *R esc.* acclimatized to room temperature.

Date.	Temperature ° C.	Condition.	Stimulus.		Initial heat, 10^{-6} cal./gm.			Total heat, 10^{-6} cal./gm.	Ratio, Total/Initial.
			Duration, sec.	Frequency per sec.	Start, per sec.	End, per sec.	Total.		
May 31	20·1	Normal	12	380	7·0	5·0	72	1410	20*
January 5	19·2	Normal	32	376	4·1	4·5	138	4230	31
January 27	18·9	Normal	32	640	6·0	4·4	166	5230	31½
February 6	19·2	Normal	24	540	6·1	6·1	147	3800	26
February 9	19·0	Normal	32	192	4·4	3·8	131	4065	31
February 15	18·6	Normal	32	474	4·5	4·5	144	4210	29
			later		7·8	4·9	203	5640	28
February 21	18·2	Normal	32	435	6·3	6·3	201	5050	25
									Mean 29
January 31	18·5	2R	32	660	5·4	3·4	141	7500	53
February 2	18·7	2R	24	600	6·3	6·3	151	7950	53
March 7	20·0	2R	24	607	5·8	5·8	139	5590	40
March 4	19·7	½R	32	395	1·75	1·65	54	2705	50
March 8	20·3	½R	32	447	2·4	1·3	59	1910	32
February 13	18·8	Sugar-R	32	420	10·7	9·9	330	8050	24
				later	9·9	9·9	317	5020	16
March 10	20·8	Sugar-R	24	640	12·1	10·0	266	5840	22
				later	9·9	7·5	209	4780	23

2R = Ringer's solution of twice, ½R = Ringer's solution of half, the normal concentration in respect of all constituents. Sugar-R = Ringer's solution with 4·5% glucose added.

* Omitted from mean; recovery followed only for 20 minutes.

is not necessarily, nor always, the case.) At the moment when the "gap" was given the total steady heat rate due to stimulation was $36 \cdot 6 \times 10^{-6}$ cal./gm. sec. The ratio, therefore, of total to initial heat was $36 \cdot 6/4 \cdot 05 = 9$.

The analysis of a "gap" is usually of the type shown in fig. 2 or fig. 3. Various other types, however, occur, of which one is shown in fig. 4, together

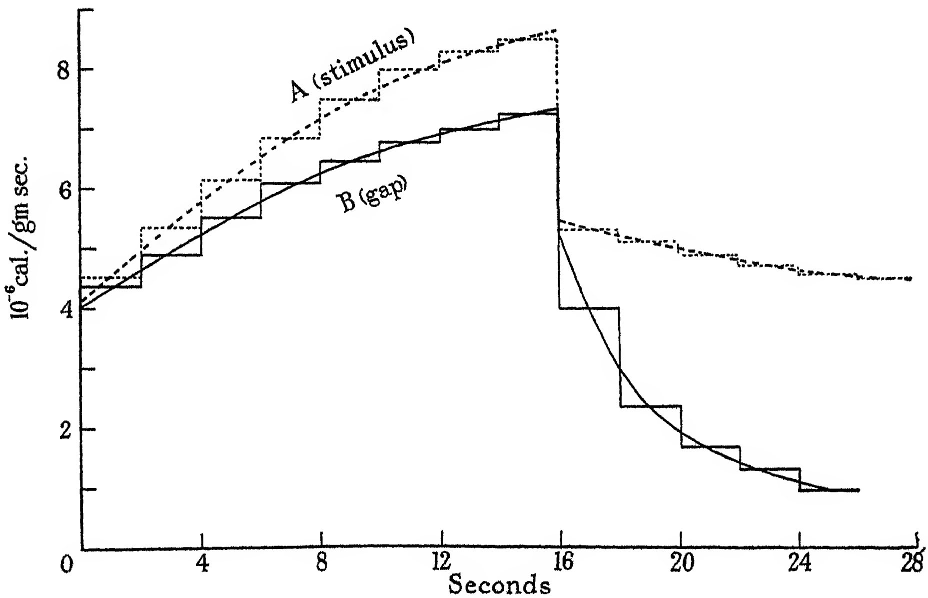

FIG. 2.—*Experiment of January* 11, 1933, at 18·9° C. Broken line, (A) analysis of heat production due to 16 seconds positive stimulus of a resting nerve, at 5-minute intervals, preceding the steady state (mean of 6); full line, (B) analysis of negative heat production due to 16 seconds negative stimulus (mean of 5 "gaps") at 5-minute intervals during the steady state. Shocks 54·7/sec. both for (A) and for the steady stimulation in which (B) refers to the "gaps."

The initial heat is: for (A) $4 \cdot 15 \times 10^{-6}$ cal./gm. sec.; for (B) $4 \cdot 05 \times 10^{-6}$ cal./gm. sec. The total heat during the steady state for (B) was $36 \cdot 6 \times 10^{-6}$ cal./gm. sec. Ratio for (B) of total to initial heat $= 36 \cdot 6/4 \cdot 05 = 9$.

Note that (B) falls much more rapidly at the end of the "gap" than (A) at the end of stimulation.

with the record from which it was calculated. There is never any difficulty in obtaining the initial heat at the moment of omitting the stimulus. In fig. 4 this is $3 \cdot 2 \times 10^{-6}$ cal./gm. sec.; the steady heat rate was 34×10^{-6} cal./gm. sec.; the ratio, therefore, of total to initial heat was $34/3 \cdot 2 = 10 \cdot 6$.

The results of 11 such measurements are given in Table III. The mean value of the ratio is $9 \cdot 1$.

There is another possible way of estimating the ratio of total to initial heat during a steady state of stimulation. Instead of comparing the initial heat rate derived from the analysis of gaps in the stimulus with the steady heat rate, we might compare it with the total amount of heat omitted due to the omission of the stimulus. From the initial sudden drop of heat rate at the

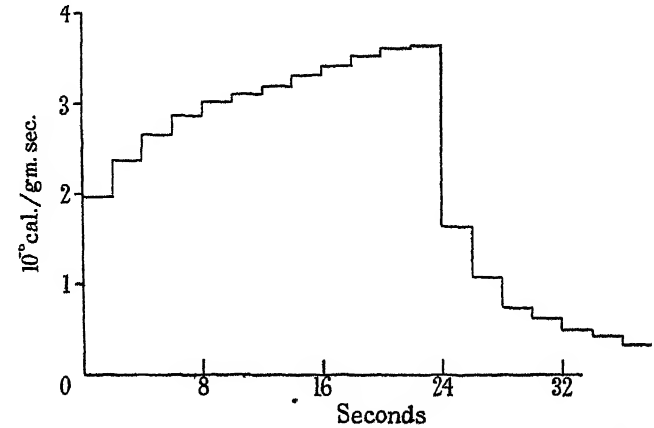

FIG. 3.—Analysis of "gap" in the heat production of nerve, due to omission of stimulus for 24 seconds during a steady state of stimulation at 27·5 shocks/sec. Mean of "gaps" at 34, 38, 42, 46 and 50 minutes from the beginning of stimulation. December 15, 1932, at 20·1° C.

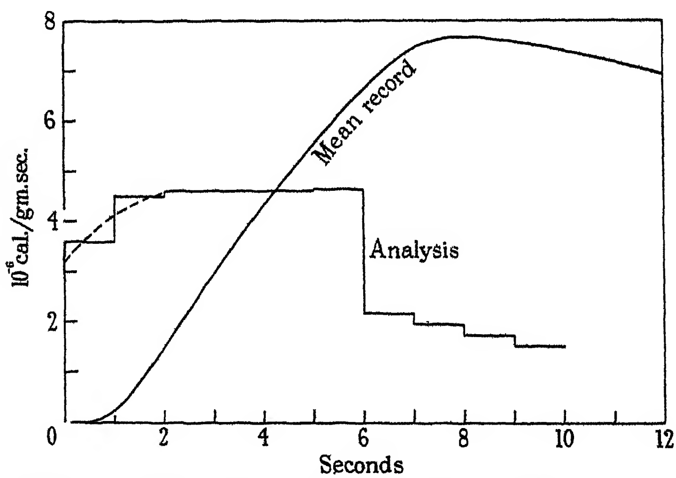

FIG. 4.—*Experiment of May* 31, 1932. Analysis of 6 seconds "gaps" in heat production of nerve during steady stimulation with 65 shocks per second. Mean of 12 records at 3 minute intervals between 34 and 72 minutes from the beginning of stimulation. Steady heat rate 34×10^{-6} cal./gm. sec. Initial heat rate from analysis $3 \cdot 2 \times 10^{-6}$ cal./gm. sec. Ratio $10\frac{1}{2}$.

Table III.—Analysis of records of gaps in steady stimulation.

Date.	Temperature ° C.	Condition.	Frequency of steady stimulation, shocks per sec.	Duration of gap, sec.	Initial heat rate, 10^{-6} cal./gm. sec.	Steady heat rate, 10^{-6} cal./gm. sec.	Ratio of total to initial heat.
May 31	19·0	Normal	65	6	3·2	34	10·6
December 15	20·1	Normal	27·5	24	1·7	15·8	9·2
January 3	19·8	Normal	23·5	16	2·1	17·1	8·1
			36·4	16	2·2	25·4	11·6
January 5	19·2	Normal	37	16	3·0	27·8	10·8
January 11	18·9	Normal	54·7	16	4·05	36·6	9·0
			about 700*	16	3·7	35·0	9·5
January 27	18·9	Normal	59	16	2·2	22·0	10·0
			183*	16	both decreasing	—	8·9
February 15	18·6	Normal	54	16	3·7	21·4	5·8
February 21	18·2	Normal	45	16	3·6	25·3	7·0
							Mean 9·1
January 31	18·5	2R	67	16	5·3	38	7·2
February 2	18·7	2R	61	16	7·6	47	6·2
March 7	20·0	2R	51	16	4·1	30·4	7·4
March 4	19·7	$\frac{1}{2}$R	54	16	1·05	18·8	18
March 8	20·3	$\frac{1}{2}$R	22	16	2·2	19·2	8·7
February 13	18·8	Sugar-R	58	32	6·2	39·3	6·3
March 10	20·8	Sugar-R	42	16	6·0	33·2	5·5

* State not steady; fatigue setting in.

beginning of the gap and its final sudden rise on resuming the stimulus at the end (say) of 16 seconds, we could estimate the average initial heat rate during the gap and so the whole amount of initial heat omitted. From the total area of the negative deflection caused by the omission of the stimulus the total heat could be calculated. The procedure indeed is precisely the same as that for finding the ratio of total to initial heat for an ordinary positive stimulus to a resting nerve. The ratio so found for 16 seconds "negative stimulation" is between 2 and 3; it is evident indeed, from figs. 2, 3, and 4, that it is bound to be very low, since the recovery heat falls off so rapidly.

We have, therefore, ratios of total heat to initial heat ranging from 2 or 3 up to 31. For each method the results are consistent; what can be the cause of the surprising differences between them? The first method is applied to nerve which has completely recovered from previous stimulation; the second and third methods to nerves which have been continuously stimulated at a moderate rate for many minutes. May nerves under the two sets of conditions actually have a different ratio of total to initial heat?

III. *The Effect of Steady Activity on the Ratio.*

When a nerve is continuously stimulated at 20° C. at any frequency lower than 60 per second, its rate of heat production reaches a steady value in about 30 minutes. Now, instead of making gaps in a constant heat rate by omitting the stimulus, it is possible to do the converse, namely, to superimpose a short high-frequency stimulus on top of a continuous low-frequency one and to use as base line the uniform heat rate due to the latter. In this way we can find the initial and the total heat for the superimposed stimulus in exactly the same way as for a short stimulus to a fresh nerve. To give the superimposed stimulus it is necessary merely to change the frequency of stimulation from low to high for a period, say, of 24 or 32 seconds. This is easily done with two commutators (see fig. 3 in the second of these papers) driven at different speeds and connected in such a way that the battery and condenser can be transferred instantly from one to the other by pressing a Morse key.

In one such experiment a stimulus of 32 seconds, at a frequency of 435/sec., was applied (*a*) during rest, and also during a steady state of stimulation at frequencies of (*b*) 2·2, (*c*) 6·6, (*d*) 17, and (*e*) 45 shocks per second respectively. The whole initial heats were (*a*) 201, (*b*) 227, (*c*) 190, (*d*) 117, and (*e*) 31 microcalories per gram. The total heats, found from the deflection-time area, were (*a*) 5050, (*b*) 4030, (*c*) 3000, (*d*) 1140, and (*e*) 227. The ratios, therefore, of

total to initial heat were (*a*) 25, (*b*) 17·8, (*c*) 15·8, (*d*) 9·7, and (*e*) 7·3. Pre-existing activity, therefore, does change the ratio of total to initial heat.

Various experiments of this kind were performed and their results are given in Table IV. The effect of activity is precisely as demanded by the discrepancy between the ratio found in the usual way and that based on gaps in the steady state. The general effect, indeed, is visible at once on the records themselves; the return of the curves towards the base line after the end of stimulation is strikingly faster for superimposed stimuli than for stimuli to resting nerve, clearly indicating greater recovery heat for the latter.

In the experiment of February 21 both superimposed stimuli and gaps were given on the base of continuous stimulation at 45 shocks/sec. As seen in Tables III and IV the ratio for the former was 7·3, that for the latter 7·0. Given the same degree of activity to operate on, it is indifferent whether the ratio is determined by positive or by negative stimulation. Thus, positive stimuli to a resting nerve and gaps in the stimulus to a continuously stimulated nerve give different ratios merely because the steady state of the nerve is different in the two cases and probably, as we shall see later, the level of recovery restoration is different also.

Another way of showing the effect of activity upon the ratio of total to initial heat is to give a very long stimulus and to estimate the average initial heat rate and the total heat in a manner similar to that described for a shorter stimulus. In one experiment in which a 32-second stimulus gave a ratio of total to initial heat of 31, a 6-minute stimulus gave a ratio of 17·5.

The difference, therefore, between "stimuli" and "gaps" is solved, but the other discrepancy remains. The initial heat as such has nothing to do with this, the difficulty is in respect of the two methods of measuring the total heat. Why, in fact, when a nerve is certainly in a steady state should the total heat omitted by omitting 16 seconds of stimulation be different from the total heat in 16 seconds as measured from the interpolated base line?

No certain answer can be given to this question, but in asking it one makes the tacit assumption that the omission of (say) 16 seconds stimulation has no other effect than to cause the omission of a certain amount of initial heat and of its consequent recovery heat. It is possible, however, that the course of the recovery from activity previous to the "gap" may be affected—perhaps quickened—by removing the necessity for recovery from activity during the 16 seconds in question. If recovery from previous activity were to take the place of the recovery which is no longer required, the total heat omitted would be made to appear much less. The analysis of a "gap" always shows (see

Table IV.—Analysis of records of superimposed stimuli.

Date.	Temperature ° C.	Condition.	Frequency of steady stimulation, shocks per sec.	Superimposed stimulus.		Initial heat of superimposed stimulus, 10^{-6} cal./gm.			Total heat of superimposed stimulus, 10^{-6} cal./gm.	Ratio of total to initial heat.
				Duration, sec.	Frequency, per sec.	Start, per sec.	End, per sec.	Total.		
February 6	19·2	Normal	18	32	540	4·8	3·3	130	1670	12·8
February 9	19·0	Normal	13½	32	448	3·7	3·3	112	1700	15·2
February 15	18·6	Normal	1·8	32	474	7·2	6·3	216	4670	21·6
			7·4	32	474	5·8	5·4	179	2745	15·3
			16	32	474	3·6	2·7	101	1290	12·8
			48	32	474	0·72	0·39	18	166	9·2
February 21	18·2	Normal	2·2	32	435	7·1	7·1	227	4030	17·8
			6·6	32	435	5·6	6·3	190	3000	15·8
			17	32	350	4·2	3·1	117	1140	9·7
			45	32	350	1·25	0·67	31	227	7·3
March 7	20·0	2R	51	24	400	4·3	2·9	86	590	6·9
February 13	18·8	Sugar-R	13	32	333	11·8	8·3	321	3785	11·8
			47	32	333	4·7	2·7	119	1010	8·5
March 10	20·8	Sugar-R	42½	24	418	6·8	5·6	149	835	5·6

figs. 2, 3, and 4) a very rapid fall at its end (actually a rise, since the heat in a gap is negative), which suggests that only the "A" process, but not the "B" process of recovery (see the first paper of this series) has been omitted. It may well be therefore that the paradox really does not exist, that we have no right to assume that after omitting the stimulus for a short period all the other processes are unaffected.

In measuring the ratio of total to initial heat by the method of "gaps" in the stimulus, it is necessary to assume that the recovery heat at any moment corresponds to the initial heat at that moment. If a steady state genuinely exists, this is correct; if fatigue is setting in, owing to too high a frequency of stimulation, the recovery heat at any moment is too large to correspond to the initial heat at that moment, but corresponds rather to the greater initial heat at some previous moment. We should expect, therefore, to find during the onset of fatigue too high a ratio of total to initial heat. Actually in Table III there are two experiments in which the frequency was too great for a steady state to be possible, but nevertheless the usual ratio of about 9 was obtained. The reason, presumably, is that the rate of fatigue was comparatively low, and of recovery rather high, the difference of phase between initial and recovery heat being insufficient to cause a perceptible change in the ratio. This gives us the greater confidence in accepting its accuracy when the state is at least approximately steady.

IV. *The Effect of Anisotonic Solutions upon the Ratio.*

The above experiments have shown that steady pre-existing activity affects the ratio so that it may vary from 31 to 6 with increasing degree of basic activity. We have looked for other conditions which might also affect it. Results have been obtained with hypo- and hypertonic solutions, which, though not yet intelligible, seem definite enough to be recorded. In Table I one experiment each was given with half Ringer ($\frac{1}{2}$R), twice Ringer (2R) and 5% CO_2. The last named is without effect. The hypo- and the hypertonic solutions, on the other hand, seemed to exert a profound influence on the ratio and (what made the matter more interesting) in opposite directions. Further experiments, therefore, were made in the expectation that dehydration or hydration might concentrate or dilute the reacting substances responsible for the heat production of recovery and so accelerate or retard the reaction and perhaps affect the ratio. The experiments with dilute ($\frac{1}{2}$R) and concentrated (2R) solutions were repeated with the improved apparatus lately available.

A summary of the results of these experiments (initial heat, total heat and their ratios) is included in Tables II, III, and IV.

Experiments were also made with a hypertonic solution consisting of the ordinary constituents of Ringer's solution in the usual concentrations plus 4·5% glucose; this has double the normal osmotic pressure. The two hypertonic solutions, sugar Ringer and twice Ringer, agreed in showing a decrease of the ratio of total to initial heat at the steady state but disagreed in that the former made the ratio for a short stimulus to the fresh nerve larger, while the latter made it smaller.

This last result puts us on our guard against the original conception that the effects of ½R and 2R are merely those of hydration or dehydration. Furthermore, nerves treated with the sugar Ringer's solution show no more depreciation at the end of 15 hours than do normal nerves, while those treated with hyper- or hypotonic Ringer always deteriorate considerably during such long survival. The effects of ½R and 2R are probably at least partly ionic, the abnormal concentration of one or other of the ions affecting the state of the nerve.

As to how the ratio of total to initial heat is changed one way or the other by anisotonic solutions it is not possible at present to say. The effect, as a matter of fact, may be rather large, as Table II particularly shows. It is of interest in confirming the conclusion that the ratio is not fixed and immutable, but can vary according to the conditions.

V. *Discussion.*

Of our values of the ratio of total to initial heat only that found in a short stimulus to a normal resting nerve, namely, about 30, can be compared with Bronk's, namely, 11. While Hill's work (1932) prepared us for a larger ratio than that given by Bronk, the discrepancy found is somewhat astonishing. It is accountable, however, when the apparatus used by Bronk is compared with that now available.

As seen in fig. 1, the recovery heat from a 32-second, or 24-second, stimulus requires more than 30 minutes for completion, and that from the shorter stimuli of 9 to 15 seconds employed by Bronk must have needed a similar time. Bronk, with the apparatus then available here, could not follow the recovery heat for more than 10 or 11 minutes. His thermostat was not constant enough to permit continuous recording over many minutes with certainty; the interior of the nerve chamber was exposed to pressure changes in the air and—what is

important in the present connection—the thermopile then used lost heat far too rapidly to give adequate information regarding the prolonged tail of the recovery heat formation. His 3-second heating control, for example, came back in about 70 seconds to its zero; with our present apparatus it ends only after about 220 seconds.

It is readily seen in fig. 1 that assuming the recovery heat to last only 10 or 11 minutes excludes more than half of it. Bronk's recovery heat, therefore, is probably less than half of the true amount. His initial heat, on the other hand, is probably a little too large; his procedure would probably leave a small amount of recovery heat in his estimate of the initial heat. The two errors together, particularly the former, explain the discrepancy.

The most interesting fact yielded by the present work is the decrease of the ratio of total to initial heat with the degree of basic activity in the steady condition on which stimulation is superimposed. What is the meaning of this change and what is its mechanism? All the evidence points to the real objective existence of the initial heat as a separate entity, and we may regard it as the index of a breakdown accompanying conduction, or perhaps the immediate recovery therefrom which is completed by the time the refractory period is over. The recovery heat represents the total energy expended in the restitution process. A decrease in the ratio would then in a sense be an increase in the "efficiency" of restitution. In what way can we picture a gain of "efficiency" as the result of steady activity?

It is a characteristic of many biological, as well as economic, processes, which is referred to in agriculture by the expression "the law of diminishing returns," that the last stage may be more expensive than the first. The same behaviour is found in many actual chemical or physical processes, *e.g.*, in the separation of two substances. A nerve which is being continuously stimulated at a low frequency, say, of 20 per second, establishes itself at a certain average level of partial activity or discharge; the restitution process has only to be such as to bring the nerve back to this level. It may well be that to effect the first 70% of full restoration—whatever that means—is relatively a much more economical process than to complete the last 30%. If this general idea be correct, the ratio of total heat to initial heat should be smaller for partially active nerve, or, referring to the results of the present paper, for superimposed stimuli, or gaps in the stimulation, during the steady state.

Fig. 5 represents a hypothetical relation between the degree of restitution and the amount of energy required to effect it. The curve describes a state of affairs in which the process of restitution becomes increasingly inefficient as

its degree approaches completion. Besides giving an intelligible description of the variation in the ratio of total to initial heat with the degree of pre-existing activity, it serves also to fit the diverse results given by the various hypo- or hypertonic solutions into a single scheme. One curve, for example, describes for half-Ringer nerve the fact that its " efficiency " is, for every degree of restoration, low relatively to that of normal nerve ; another for sugar-Ringer nerve, that its efficiency is greater throughout the whole range than for normal nerve ; a third, for twice-Ringer nerve, that for restoration to complete rest

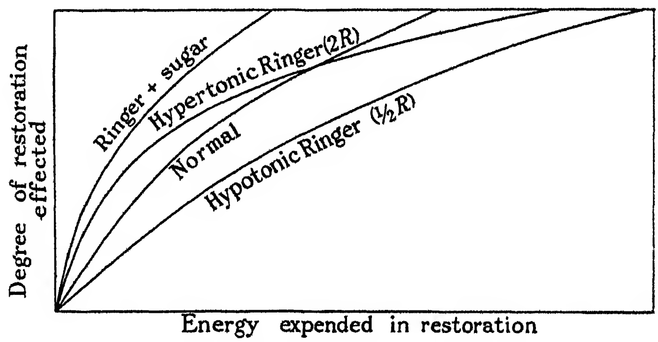

FIG. 5.—Diagram to illustrate the hypothesis of " diminishing returns " in the restoration of nerve from stimulation to a standard state. The horizontal line at the top represents the condition of a nerve restored to complete rest, a horizontal line at any other height would represent the degree of restoration to some steady level of activity. Each curve represents the relation between a given level of restoration and the amount of energy required to return to that level when displaced by a given stimulus.

its efficiency is lower, but for restoration to a state represented by a moderate degree of activity its efficiency is greater than for normal nerve. While this diagram affords no explanation of the cause of the phenomena, it allows them to be fitted into a reasonable and intelligible scheme.

One general conclusion from the whole of this discussion is that the recovery process is not rigidly coupled to the initial process, as it would be if both were parts of an invariable sequence of chemical events. It would seem rather that when a nerve has been active, various changes—perhaps physical in nature —have occurred either during conduction, or during the refractory period, or both, and these have to be reversed with the liberation of energy by oxidation in a prolonged and separate process during the following 30 minutes (at 20° C.). We can refer to the model, many times used in this connection for muscle, of an electrical storage battery which runs down during activity and is recharged by a separate mechanism afterwards. The amount of energy used by the

motor which drives the dynamo, by which recharging is effected, may not be directly proportional to the energy released by the storage battery during discharge.

The conclusion is supported by the results of experiments on crab's nerves (Beresina and Feng, 1933). Here the ratio of total to initial heat is still higher, namely, 40 to 70. This is for stimuli applied to a completely resting nerve returning during recovery to a state of complete rest. The range of values found by these authors nearly overlaps at its lower end the range obtained in the present work with frog's nerve at its upper end. It is unlikely that the chemical reactions in nerve activity are fundamentally different in the two cases, so that we have values lying between 6 and 70 for the ratio of total to initial heat. No variation of this kind has ever been found with muscle, where the ratio is, in fact, rather constant. In muscle, recovery may be a chemical consequence of the breakdown during contraction ; in nerve it seems rather to be involved in a restoration, to some level depending upon the circumstances, of something analogous to a difference of potential, or perhaps of concentration.

Summary.

(1) After a short stimulus (*e.g.*, 16 to 32 seconds) to a completely resting frog's nerve at 20° C. the recovery process is complete only in 30 to 35 minutes. The ratio of total heat to initial heat is about 30.

(2) When a nerve is stimulated continually at not too high a frequency a steady state is reached in about 30 minutes in which the rate of heat production (above "basal") is constant. If the stimulus be omitted for a short time (*e.g.*, 16 seconds) a "gap" in the heat production occurs which can be analysed in the usual way. This provides a rather accurate estimate of the initial heat during the steady state. The ratio of the total heat to the initial heat so measured is about 9.

(3) A nerve in a steady state of activity can be stimulated to a higher degree of activity by increasing the frequency of stimulation for a short time (*e.g.*, 32 seconds). The heat so produced can be analysed in the usual way, treating the level of steady activity as a base line. The ratio of total to initial heat, for the superimposed stimulus, may vary from 30 to 7, depending on the level of the basal activity, being less the greater that activity.

(4) The nearer the basal steady condition of a nerve is to one of complete rest the higher is the "potential" to which it is restored during recovery, and the greater is the amount of energy used in its restoration.

(5) Hypertonic and hypotonic solutions affect the ratio of total to initial heat in a characteristic way.

(6) The wide variation, in nerve under different conditions, of the ratio of total to initial heat suggests that the initial and the recovery processes are not directly coupled (*e.g.*, chemically); the former is doubtless the consequence of the latter, but the efficiency, or degree, of recovery varies with the circumstances.

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The Effect of Veratrine on the Heat Production of Medullated Nerve.

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Graham and Gasser (1931) have recently investigated the effect of veratrine upon the electrical response of frog's medullated nerve. The prolonged after-potentials which they observed led Professor Gasser to suggest that it would be interesting to examine also the effect on the heat production. In muscles (Hartree and Hill, 1922) the prolonged veratrine contracture is associated with a prolonged evolution of heat of the same order of size as in a tetanus developing the same force. It has been found that in nerve also, when fully veratrinized, a prolonged production of heat occurs in response to a single shock, and that the total amount of this heat may be many hundreds of times as great as that in the response of a normal nerve.

Graham and Gasser used *R. pipiens*, I have used autumn Hungarian *R. esc.* They made up their veratrine in solutions of suitable strength and applied it to the nerve with a brush. The necessity of thermal equilibration demanded that my nerves should be soaked. I have used veratrine hydrochloride in Ringer's solution buffered with phosphate to p_H 7·2, of concentration about 1/50,000, and the nerves were usually soaked in this on the thermopile for about 1 hour.

Graham and Gasser found no effect of veratrine on the initial "spike" of the action potential; the effect is only on the after-potential, which lasts much longer than usual. According to them even 500 σ after the shock the after-potential may be as great as 1% of the "spike" height, and their records "suggest that an interval considerably longer than 1 second frequently elapses before the after-potential reaches the zero line." These, as it proves, are very conservative estimates: the action current (as will be shown by Fromherz (1933) elsewhere) and the heat production persist, not for a few seconds, but for several minutes in a nerve effectively poisoned with veratrine.

Graham and Gasser found that the immediate effect of previous stimulation was to increase the after-potential produced by a shock. With rest the after-potential diminished again. This is the exact contrary of what we have found—in order to obtain the greatest response, whether in heat or in action current, from a fully veratrinized nerve it is necessary to give it a long preceding rest. For muscle also the veratrine response is seen at its best after a long interval free from stimulation. The facts are evident enough on both sides; the contradiction must be due to differences of treatment or material.

In the first few experiments no considerable difference was found between veratrinized and normal nerve. The heat produced in response to a single shock was too small to detect. There was indeed some evidence of a change; for example—

(A) *Effect of Frequency of Stimulation.*—In a 6-second stimulus half the maximum heat was obtained at a very low frequency, about 15/sec. instead of the 80/sec. characteristic of a normal nerve; if its impulses last longer we should expect a nerve to function within a lower range of frequency.

(B) *The Analysis of the Heat.*—The "initial" heat was exceptionally large at the beginning and there was no sharp distinction between initial and recovery heat at the end of stimulation; it seemed as though the initial heat stopped gradually and not suddenly when the stimulus ended. If this, however, were all, the veratrine effect in nerve was insignificant compared with that in muscle.

The same absence of any considerable effect was found for the action current measured with a sensitive moving magnet galvanometer. The instrument of course could not separate the "spike" and the immediate after-potential, as the oscillograph used by Graham and Gasser was able to. It could, however, give an accurate picture, which their instrument could not so easily do, of any

slow potential changes occurring after (*say*) $1\frac{1}{2}$ second from the shock. As Fromherz describes elsewhere, there was no very obvious sign, after soaking in 1/50,000 veratrine for an hour, that the nerve had been poisoned at all.

In these preliminary experiments the nerves had been in oxygen or in oxygenated Ringer's solution throughout; those of Graham and Gasser had been presumably in air. A rather large isolated nerve in air, particularly if in contact with electrodes on one side, is always in danger of partial asphyxiation. Assuming a resting heat production of 70×10^{-6} cal. per gram per second, as found by Beresina (1932) at 20°, and the diffusion constant found for muscle by Krogh (1918) it can be calculated (Hill, 1928, p. 60) that the critical diameter is about $2\frac{1}{2}$ mm. If the nerves were exposed to air on one side only the critical diameter would be about one half of this. If the temperature were higher, and so the resting metabolism greater, the critical diameter would be less.* The diffusion constant in nerve (which is known to be rather impermeable to some substances) may very well be less than that of muscle; and the rate of oxygen consumption is increased by stimulation. There is no security, therefore, that a nerve of 1 mm. diameter in contact with electrodes will be fully supplied with oxygen if it be surrounded by air. It is safer, at any rate, to place it in oxygen.

Graham and Gasser had shown that during recovery from partial asphyxia (40 minutes in commercial nitrogen) the after-potential is considerably greater than initially. Fromherz (1933) has found that in veratrinized nerve about $1\frac{1}{2}$ hours are needed for complete asphyxia; the effect of partial asphyxia suggests that a certain degree of oxygen want in the interior of the nerve in air may have affected Graham and Gasser's results and that stimulation may have produced the effect that they noticed by adding to the degree of oxygen want. The fact that our nerves were in oxygen may have prevented them from showing the partial effect of veratrinization which Graham and Gasser found. Complete veratrinization is a much more considerable affair.

The asphyxia of the nerves in Graham and Gasser's experiments was incomplete. A very striking effect, however, is produced by complete asphyxia and recovery. A nerve previously soaked for an hour in 1/50,000 veratrine is kept in pure nitrogen or hydrogen for 3 hours until no trace of response occurs on stimulation. Oxygen is then admitted, and within a few minutes the veratrine effect is shown at its best. The action potential, or perhaps one should say the after-potential, produced by a single shock and recorded by a

* According to Schmitt, quoted by Gerard (1932, p. 540), nerves treated with veratrine show an increased oxygen consumption.

sensitive galvanometer, goes through a series of changes which is strikingly similar to the contracture of a veratrinized muscle. The discharge is hundreds of times as great as in a normal nerve, or as in a nerve poisoned without asphyxiation, and it may last many minutes. This typical veratrine effect has the further likeness to that in muscle that it is rapdily fatigued but recovers when the tissue is allowed to rest. It is in nerve treated in this way that the heat production has been measured.

Method.—All experiments were on the sciatic nerve of Hungaria *R. esc.* at about 19° C. The nerves were placed on the thermopile and immersed in buffered Ringer's fluid (p_H 7·2) containing about 1/40,000 veratrine (1/20,000 to 1/50,000). Oxygen was bubbled and after about 1 hour the solution was removed and replaced by pure nitrogen. After about 2 hours the heating controls for the analysis were made on the now inexcitable nerve. After 3 hours the nitrogen was replaced by oxygen. A large heat production occurred (corresponding—see Beresina, 1932—to the oxygen debt paid off) and after a further hour the heat production in response to a shock could be measured. Considerable intervals were allowed between stimuli to permit the veratrine effect to be manifested at its best.

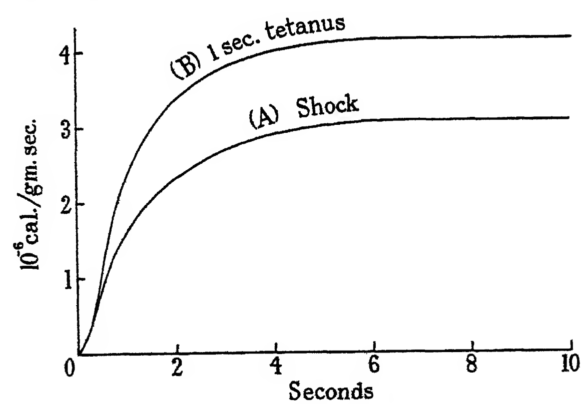

Fig. 1.—Interpolated curves from the analysis in 1-second units of the heat production of frog's medullated nerve treated with veratrine. Curve A, single shocks; curve B, 1-second tetanus.

Results.—In fig. 1 curves are given interpolated through the results of an analysis in 1-second time units of the heat produced (A) in response to a single shock, and (B) in response to a 1-second tetanus, of a nerve treated with veratrine as described above. (A) refers to the mean of records taken at intervals of 15 minutes; with a shorter interval of 7 minutes the curve was of the same form but of only two-thirds the height. (B) was after a long rest.

The rate of heat production rises gradually in either case to a maximum. The heat is not "initial" heat in the ordinary sense; the analysis is quite unlike that from an ordinary stimulus. From its maximum the heat production falls only slowly. In fig. 2 analyses are given of the rate of heat production in two other experiments, (A) for a single shock, (B) for a 1/5-second tetanus. In (A) the total heat in the first minute is more than 100 microcalories per gram; in (B) it is nearly 180. In a previous paper it was shown that during a steady state of stimulation at low frequency the total heat per impulse is of the order of 1 microcalorie per gram. In (A) the total heat including recovery must have been many times the 100 microcalories of the first minute; probably at least 1000. Thus the heat liberated by a single shock in a veratrinized nerve may be of the order of 1000 times as great as in a normal nerve.

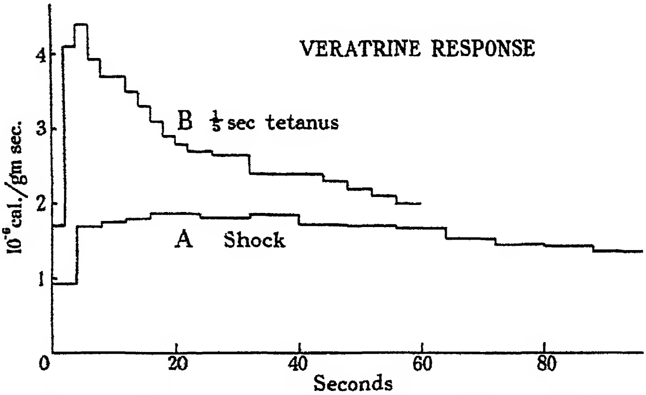

FIG. 2.—Analysis in 4- and 2-second units of the heat production of nerve treated with veratrine. A, single shocks after long rest; B, 1/5-second tetanus at 28 shocks/sec. after 43 minutes rest.

If the interval between stimuli is less than about 20 minutes the veratrine effect is reduced, but even with 1-second intervals it is still obviously present. In one experiment a veratrinized nerve was stimulated at the rate of 30 shocks per minute for 10 minutes. In this time, of course, a steady state was not obtained, though there were signs that it would have been had stimulation been continued. The rate of heat production already reached was 17 microcalories per gram per second, 34 microcalories per gram per impulse; this is more than thirty times as great as one would expect in a normal nerve. Fig. 3 shows the way in which the rate of heat production obtained by analysis began to rise in another experiment during stimulation at 30 shocks per minute; at 60 shocks per minute the curve was similar but 25% higher, doubling the frequency had increased the heat production only by one-quarter.

In fig. 4, in still another experiment, the analysed rate of heat production is shown at the beginning of stimulation (A) with one shock every 8 seconds, (B) with one shock every 4 seconds, and (C) with one shock every 1 second. The heat production does not increase nearly in proportion to the frequency of stimulation ; the heat per impulse, therefore, is greater the longer the interval between impulses.

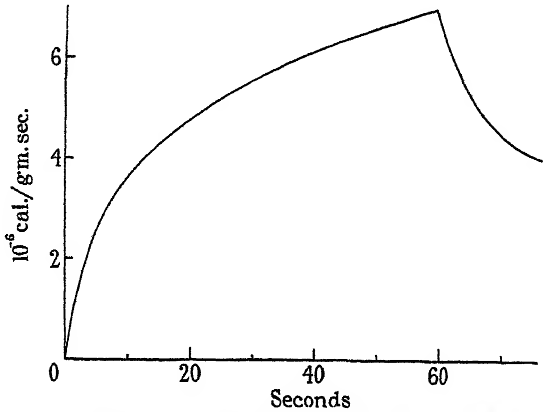

FIG. 3.—Analysis of heat production in 4-second units during and after 1 minute stimulation at 30 shocks/min. With 60 shocks/min. the curve was similar but 25% higher.

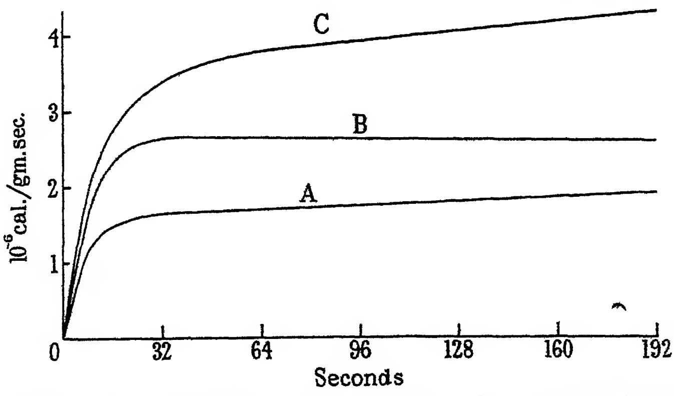

FIG. 4.—Analysis of heat production, in 16-second units, of nerves treated with veratrine during prolonged stimulation, A with 1 shock in 8 seconds, B with 1 shock in 4 seconds, and C with 1 shock every 1 second.

An experiment was made with regular stimulation at intervals of 1, 2, 4, 6 and 10 seconds. At 50 seconds after the start the following rates of heat production were found by analysis :—

Interval, seconds..............	1	2	4	6	10
Rate of heat production, 10^{-6} cal./ gm. sec.	4·7	3·9	3·2	2·3	1·75

Judging from other experiments a single shock at time zero would have caused a rate of heat production at 50 seconds of at least 10^{-6} cal./gm. sec. The extra 49 shocks at 1 per second have only produced an extra $3 \cdot 7 \times 10^{-6}$ cal./gm. sec.

It is clear, therefore, that the veratrine effect requires a considerable interval for "recovery." For muscle the veratrine-recovery is complicated by muscular fatigue; with nerve there is no evidence of fatigue, the nerve can still give its normal response to a stimulus. It is only the abnormal veratrine response which needs time for its restoration. There is no clue as to what the mechanism of this behaviour can be, but it seems that something needs to be restored before the veratrine response can occur to its full again.

The question why a medullated nerve needs to be asphyxiated after poisoning, before veratrine can produce its full characteristic effect, is dealt with elsewhere by Fromherz (1933). On non-medullated nerve veratrine acts without asphyxiation and in much lower concentrations (Cowan, 1933). The simplest hypothesis is that veratrine cannot penetrate the myelin sheath of the nerve fibre until this is somehow rendered permeable by oxygen want.

Summary.

Frog's medullated nerves treated with 1/50,000 solution and kept in oxygen show little effect of veratrine on the heat production. If, however, after treatment, they be completely asphyxiated and then allowed to recover, a striking and characteristic effect is obtained. The heat production in response to a single shock rises in a few seconds to its maximum rate; in amount it may be 1000 times normal; it may last many minutes. The heat production during slow stimulation may be greatly increased.

The veratrine effect requires time for its restoration; after a stimulus it is not shown again to its full without a long period of rest. It is quite evident, however, with shocks even at 1-second intervals.

This series of papers owes much to various colleagues: to Mr. W. Hartree, Mrs. M. Beresina, Dr. H. Fromherz and Mr. Donald Scott, who took part in the experiments and calculations; to Professor H. S. Gasser who suggested the veratrine experiments; to Mr. A. C. Downing who constructed the thermopiles and commutator; and particularly to Mr. J. L. Parkinson for his continual loyal assistance and his construction of the thermostat.

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On the Combination of Methæmoglobin with H_2S.

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(From the Molteno Institute, University of Cambridge.)

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(1) *Hoppe-Seyler's Sulpho-derivative of Hæmoglobin.*

Seventy years ago Hoppe-Seyler (1863) found that if a current of H_2S is passed through a solution of oxyhæmoglobin a distinct absorption band appears in the red end of the spectrum and the compound turns gradually green. He found further that this change takes place only when H_2S acts in presence of oxygen, and the compound thus obtained he described as sulpho-methæmoglobin. Similar results have been obtained by several other workers and especially by Lewisson (1866) and by Araki (1890) who have shown, moreover, the co-existence of the absorption bands of sulpho-hæmoglobin with those of oxy- or reduced hæmoglobin. Harnack (1899), to whom we owe a careful revision of the whole literature of the subject, repeated these observations but claimed to obtain the appearance of the absorption band in the red end of the spectrum by passing H_2S through a solution of reduced hæmoglobin kept under a layer of paraffin, believing the reduced hæmoglobin to be thus sufficiently protected from oxygen. He concluded that "sulpho-hæmoglobin" can be obtained in complete absence of oxygen, although he agrees with Hoppe-Seyler that only in the presence of oxygen is hæmoglobin transformed by H_2S into the green compound.

More recently Haurowitz (1926) described as "sulpho-hæmoglobin" a green compound which he has obtained from hæmoglobin by passing through

it at frequent intervals during a whole week currents of H_2S and of oxygen. He described also methods of obtaining the crystals of this compound, showing the absorption band at 618 mμ. Treated with alkali and $Na_2S_2O_4$ this compound gave an ordinary proto-hæmochromogen. The porphyrin obtained from this compound did not differ from protoporphyrin in its solubilities and absorption spectrum. These facts indicated that prolonged treatment with H_2S and O_2 does not affect the prosthetic portion of the hæmoglobin molecule. The analysis of crystals gave 16 atoms of sulphur per each atom of iron, a result which seems to indicate that most, if not all, of the sulphur found was adsorbed to the crystals.

There is no doubt that the compound described by Haurowitz is identical with those obtained by previous workers and that this compound is formed only when hæmoglobin is treated by H_2S in presence of oxygen. This statement is not contradicted by the results of Harnack mentioned above, because it is now well known that a fluid covered by a layer of paraffin is not protected from the access of oxygen.

That the presence of oxygen is necessary for the production of this compound can be shown by the following experiment. A solution of fresh oxyhæmoglobin is completely reduced by boiling it *in vacuo* in a Thunberg tube, which is then filled with H_2S free from oxygen. The contents of the tube (although well shaken with H_2S) do not reveal the characteristic band in the red end of the spectrum. Only after several hours does a faint band appear in this region, owing probably to the action of some traces of oxygen which were not completely removed. This band does not become stronger even after 24 hours. On opening the Thunberg tube and shaking its contents with air a very distinct band appears almost immediately at 618 mμ and while this band becomes stronger the bands of oxyhæmoglobin gradually fade away.

It is important to note that this compound can be obtained only from hæmoglobin, oxyhæmoglobin and methæmoglobin, the latter being previously reduced by H_2S to hæmoglobin, but not from compounds such as hæm, hæmatin, hæmochromogen or parahæmatin.

The solution of hæmoglobin treated with H_2S and O_2, left standing overnight, filtered and cleared from the free H_2S by a current of air or boiled in a vacuum shows still the characteristic band at 618 mμ.

On the addition of a little hydrogen peroxide or potassium ferricyanide to the solution, this band disappears but reappears on reduction with $Na_2S_2O_4$. This clearly shows that the compound obtained from hæmoglobin, treated with H_2S and O_2 and showing a strong absorption band at 618 mμ, is a ferrous

compound which can be oxidized and reduced and in which protohæm is still combined with globin.

It is, however, impossible to say what is the nature of this compound. We do not know yet whether the globin, during treatment with H_2S and O_2, underwent some irreversible modification or whether the compound still contains the unmodified hæmoglobin combined with an oxidation product of H_2S, such as sulphur. We have not yet succeeded in recovering from this compound the original unmodified hæmoglobin. Although the nature of this compound is still undetermined, it is clear that it does not correspond to a simple combination formed of H_2S with either hæmoglobin or methæmoglobin and it does not belong therefore to the category of reversible compounds such as are formed between hæmoglobin or methæmoglobin and O_2, CO, KCN or KF.

(2) H_2S-*methæmoglobin Compound.*

(*a*) *Spectroscopic Study of* H_2S-*methæmoglobin Formation.*—In the course of this investigation it was found, however, that H_2S forms a definite compound with methæmoglobin analogous to cyanmethæmoglobin, a compound from which unmodified methæmoglobin can be easily recovered. This compound is entirely different from what was previously described by Hoppe-Seyler, Harnack, Haurowitz and others as sulpho-hæmoglobin, or sulpho-methæmoglobin, obtained by the action of H_2S and O_2 on hæmoglobin. The existence of the real H_2S-methæmoglobin compound can be demonstrated in the following way.

Methæmoglobin solution obtained by the action of potassium ferricyanide on pure hæmoglobin is dialysed (for several days) against distilled water at 0° C. until all the ferricyanide is removed from the solution. Diluted with acetate buffer of p_H 5·6–5·8 it shows a characteristic brown colour and a distinct absorption spectrum of acid methæmoglobin. On the addition of a drop of water saturated with H_2S, the methæmoglobin solution, although remaining distinctly acid, changes its colour rapidly from brown to red, and its absorption spectrum, instead of the characteristic band in the red end of the spectrum, shows on examination with a small dispersion spectroscope two diffuse bands: α-band narrow and faint at 578 mμ and β-band wider and stronger at 545 mμ. That the rapid change of colour and absorption spectrum of acid methæmoglobin is not due to the partial appearance of oxyhæmoglobin is easily demonstrated by the following facts:—

(1) The absorption spectrum, when examined with a small dispersion spectroscope, has only a faint resemblance to that of oxyhæmoglobin. The position and relative strength of the bands are entirely different.

(2) The same results are obtained when methæmoglobin is treated with H_2S in complete absence of oxygen.

(3) CO has no effect on its absorption spectrum.

The absorption spectrum of our compound differs also, as will be shown, from that of alkaline methæmoglobin. Moreover, the H_2S-methæmoglobin compound in this experiment is formed between p_H 5·4 and 6, which is far below the range of the possible existence of alkaline methæmoglobin. In fact, from the work of Haurowitz (1924) we know that methæmoglobin solution at p_H 6 exists only in the acid form, alkaline methæmoglobin begins to appear only at p_H 7, at p_H 8·4 its concentration reaches 50%, and only at p_H 10 is the conversion into alkaline methæmoglobin complete. On the addition of lead acetate to the solution of H_2S-methæmoglobin, from which the excess of H_2S was removed by boiling in a vacuum, the compound is rapidly dissociated and converted into acid methæmoglobin. The solution of acid H_2S-methæmoglobin, free from excess of H_2S, can be easily reduced with Stokes' solution to hæmoglobin, which on shaking with air gives oxyhæmoglobin.

The absorption spectrum of H_2S-methæmoglobin differs from those of acid and alkaline methæmoglobin and this can be easily ascertained by a simple examination of these compounds with an ordinary hand spectroscope. The real difference in their absorption spectra can be determined, however, only by a spectrophotometric measurement.

For this purpose, from a strong solution of methæmoglobin three dilute solutions are prepared: (1) a distinctly acid solution of p_H 5·8–6; (2) a distinctly alkaline solution of p_H 10; and (3) an acid solution to which a drop of a strong solution of Na_2S is added, sufficient to transform it completely into H_2S-methæmoglobin without producing a noticeable change in its p_H. The absorption spectra of these solutions were determined in the usual way by means of a Hilger Nutting spectrophotometer and the results obtained are shown in fig. 1. In this figure the absorption coefficient is defined as $\frac{1}{cd} \ln \frac{I_0}{I}$, where c is the concentration of methæmoglobin in gram-atoms of iron per cubic centimetre, d is the depth of the cell in centimetres, I_0 and I are the intensities of the incident and transmitted light which as $\log_{10} I_0/I$ are read off directly on the instrument.

The absorption curve of H_2S-methæmoglobin differs from that of alkaline methæmoglobin by having a stronger β-band and an almost complete disappearance of α-band, which is replaced by a kind of plateau on the long wave-

side of β-band, fig. 1. When H_2S-methæmoglobin compound is examined with a small dispersion spectroscope the flat portion of the absorption curve gives the impression of a weak and diffuse α-band lying at about 578 mμ. The absorption curve from 580 mμ upwards in H_2S-methæmoglobin is much steeper than in alkaline methæmoglobin which makes this compound in the red end of the spectrum more transparent, so that in the mixture of H_2S-methæmoglobin and acid methæmoglobin the long wave absorption band of the latter (at 635 mμ) stands out clearly.

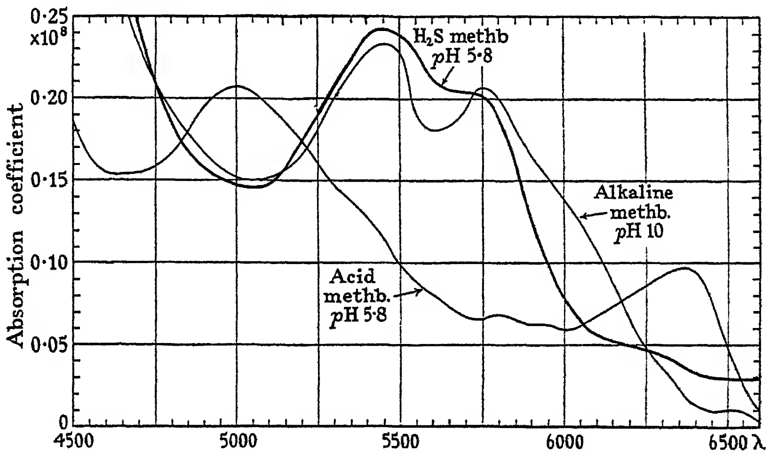

FIG. 1.—Absorption spectrum of H_2S-methæmoglobin compared with those of alkaline and acid methæmoglobin. Ordinate, absorption coefficient per gram atom of methæmoglobin iron; abscissa, wave-length in Angström units.

In order to show the minimum amount of H_2S which is required to convert the acid methæmoglobin into H_2S-methæmoglobin, the following method has been used:—Acid methæmoglobin and Na_2S were mixed in various concentrations in reaction vessels. The relative amounts of free acid methæmoglobin and H_2S-methæmoglobin formed in these vessels were determined by comparing the absorption spectra of the mixture with that given by the two substances, acid methæmoglobin and H_2S-methæmoglobin, in equal concentrations but kept separately in the two compartments of a double wedge trough.

The examination was carried out by means of a Zeiss' spectroscopic ocular attached to a microscope. The most convenient reaction vessels for thicknesses of fluid below 20 mm. are the special vessels of Zeiss' comparative spectroscope, provided with movable plungers and giving accurate readings of thicknesses to 0·02 mm. For thicknesses above 20 mm. these vessels are replaced by ordinary glass tubes with flat bottoms. The reaction vessels, with

the metal portion of the plungers protected with paraffin, were placed on the microscope stage. The double wedge trough, 150 mm. long and 23 mm. wide (inside measurements) was placed in front of the aperture of a comparison prism (after removal of the mirror and tube attachment) on a brass platform 35 cm. long, attached by means of a ring and a screw to the tube of the microscope. The position of the trough was read off on a scale carefully marked on paper, which is fixed to the platform covering its whole surface. It is convenient to protect the paper by a strip of glass upon which the trough can easily slide in front of the aperture of the microspectroscope. Both the trough and the reaction vessels were illuminated by a 4-volt bulb, the intensity of which can be varied by means of two independent resistances. The bulb which illuminates the reaction vessel was placed directly under the condenser of microscope stage. The light during the observation must be adjusted in such a way that in only one position of the trough, as it is moved along the scale, both absorption spectra match each other over the whole length of the spectrum. The reading of this position of the trough enabled us to calculate the relative concentrations of both compounds in the reaction vessel.

To avoid any complication which may be due to the dissociation of H_2S-methæmoglobin compound, in the presence of the minimum amount of H_2S, the observations must be made with fairly strong concentrations of acid methæmoglobin.

The solutions of methæmoglobin were prepared from a strong (11·6%) solution of pure methæmoglobin diluting it with acid phosphate buffer. Instead of H_2S a solution was prepared from crystals of Na_2S, $9H_2O$ (240·2) dried previously with filter paper. Na_2S when mixed in the proportions described below with methæmoglobin liberates the equivalent amount of H_2S.

A typical experiment was carried out in the following way. (The concentrations of methæmoglobin throughout this paper are expressed in terms of iron or of hæmatin, that is in terms of equivalent weight.) Both compartments of the trough were filled with acid methæmoglobin $1 \cdot 36 \times 10^{-4}$ (gm. atom of iron) and one of them in addition to it received 0·2 c.c. of 10^{-1} M of Na_2S, which transforms it completely into H_2S-methæmoglobin. The three solutions for the reaction vessels were first prepared in the following way. In each of them to 4 c.c. of $5 \cdot 44 \times 10^{-4}$ solution of acid methæmoglobin were added different amounts, such as 0·82 c.c., 0·54 c.c., and 0·27 c.c., of 4×10^{-3} M of Na_2S, each solution being made up to 5 c.c. with distilled water.

The final concentrations of the reagents and the results are shown in Table I, the reagent vessels being adjusted to a thickness of 7·2 mm.

Table I.

Methæmoglobin as Fe gm. atom per l.	H_2S, M.	% methæmoglobin.	% H_2S-methæmoglobin.	H_2S/Fe.
$4 \cdot 35 \times 10^{-4}$	$6 \cdot 6 \times 10^{-4}$	0	100	1·5
$4 \cdot 35 \times 10^{-4}$	$4 \cdot 35 \times 10^{-4}$	13	87	1
$4 \cdot 35 \times 10^{-4}$	$2 \cdot 17 \times 10^{-4}$	53	47	0·5

This clearly shows that, at the dilution of reagents used in this experiment, the minimum amount of H_2S required to transform acid methæmoglobin into H_2S-methæmoglobin corresponds to 1 molecule of H_2S per atom of iron of methæmoglobin. The exact amount of H_2S required can be determined, however, when we find the extent to which H_2S-methæmoglobin dissociates at various dilutions.

For this purpose two series of experiments have been carried out. In the first series methæmoglobin (in terms of Fe) and Na_2S have been used in equal concentrations $\frac{Na_2S}{\text{Methb.}} = \frac{1}{1}$. , in the second series the concentrations of Na_2S were exactly half that of methæmoglobin, *i.e.*, $\frac{Na_2S}{\text{Methb.}} = \frac{1}{2}$. The details of these experiments and the results obtained are shown in Tables II and III and in fig. 2.

Table II.—Methæmoglobin in trough as gm. atom Fe per litre = $6 \cdot 6 \times 10^{-5}$; $\frac{Na_2S}{\text{Methb.}} = \frac{1}{1}$.

Methæmoglobin as Fe and Na_2S.	$\log_{10}$ of molecular dilution of Na_2S.	Depth in mm. of reaction vessels.	% H_2S-methæmoglobin.	$K = \frac{[\text{Methb.}]\,[H_2S]}{[H_2S\text{-Methb.}]}$
56×10^{-5}	3·25	2·7	91	$0 \cdot 50 \times 10^{-5}$
14×10^{-5}	3·85	10·8	79	$0 \cdot 80 \times 10^{-5}$
7×10^{-5}	4·15	21·7	66	$1 \cdot 22 \times 10^{-5}$
$2 \cdot 8 \times 10^{-5}$	4·55	54·0	43	$2 \cdot 13 \times 10^{-5}$
$1 \cdot 9 \times 10^{-5}$	4·77	80·0	36	$2 \cdot 17 \times 10^{-5}$

$\log_{10}$ of reciprocal of concentration.

These experiments corroborate the results previously obtained and show definitely that one molecule of H_2S per atom of Fe is required in order to transform acid methæmoglobin into H_2S-methæmoglobin compound.

Table III.—Methæmoglobin in trough as gm. atom Fe per litre $= 6 \cdot 6 \times 10^{-5}$; $\frac{Na_2S}{Methb.} = \frac{1}{2}$.

Methæmoglobin concentration gm. atom Fe per l. *a*.	Na_2S. molarity.	$\log_{10}$ of mol. dilution Na_2S.	Depth in mm. of reaction vessels $= \frac{23 \times 6 \cdot 6 \times 10^{-5}}{a}$.	Percentage of H_2S-methæmoglobin.
112×10^{-5}	56×10^{-5}	3·25	1·35	49
56×10^{-5}	28×10^{-5}	3·55	2·7	47
14×10^{-5}	7×10^{-5}	4·16	10·8	41
7×10^{-5}	$3 \cdot 5 \times 10^{-5}$	4·46	21·7	33
$2 \cdot 8 \times 10^{-5}$	$1 \cdot 4 \times 10^{-5}$	4·85	54·0	22
$1 \cdot 9 \times 10^{-5}$	$0 \cdot 95 \times 10^{-5}$	5·02	80·0	15

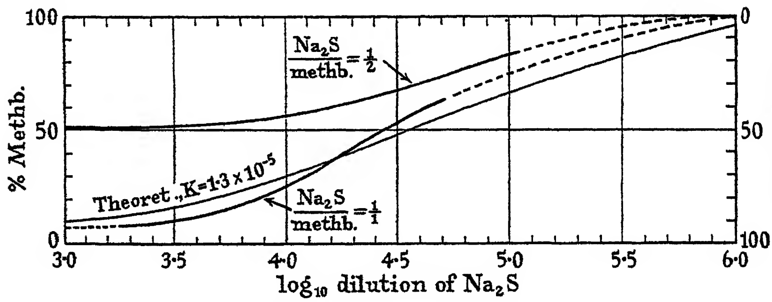

FIG. 2.—Dissociation curves of H_2S-methæmoglobin.

The dissociation constant $K = \frac{[\text{Methb.}]\,[H_2S]}{[H_2S\ \text{Methb.}]}$ of this compound is probably between $0 \cdot 8 \times 10^{-5}$ and $1 \cdot 3 \times 10^{-5}$. In fact the theoretical curve corresponding to the latter constant when drawn on the same figure passes near our experimental curve.

(*b*) *Manometric Investigation of* H_2S-*methæmoglobin Formation.*—The formation of a compound between H_2S and acid methæmoglobin can be studied not only by the spectroscopic method described above but also by means of a manometric method. The principle of this method consists of the liberation in one of the flasks of the Barcroft differential manometer of a definite amount of H_2S in the presence of either hæmoglobin or methæmoglobin solutions. The amount of the bound H_2S is found from the difference between the amount of total H_2S and the amount of the free H_2S. The latter (X_{H_2S} in mm.^{3} at N.T.P. and dryness) can be calculated from manometric reading (*h*) multiplied by the constant (K_{H_2S}) of the apparatus for H_2S.

This constant is obtained from the formula of Warburg, slightly modified by taking into consideration the angle (Q) of the manometer with the vertical and simplified by the omission in his formula of the term $V_F' \alpha'$, representing the gas mixture dissolved in the fluid of the compensating flask. The formula simplified in this way is as follows:

$$K = \left(\cos Q + \frac{A \, . \, P_0}{2V_G'}\right)\left(\frac{V_G(273/T) + V_F\alpha}{P_0} + \frac{A}{2} \cdot \frac{273}{T}\right),$$

where

Q = angle of manometer with vertical.

A = cross-section of manometer (mm.^{2}).

T = absolute temperature.

P = 760 mm. of Hg in mm. of paraffin (sp. g. 0·788) $= \frac{760 \times 13 \cdot 6}{0 \cdot 788}$.

V_G = gas volume and V_F liquid volume in experimental flask (mm.^{3}).

V_G' = gas volume in compensating flask.

α = solubility of H_2S at 20° C. 2·58 (c.c. of H_2S at N.T.P. dissolved in 1 c.c. of liquid in equilibrium with the gas, at the partial pressure of 760 mm. of Hg).

Having determined the constant for H_2S, the experiments were carried out in the following way:—312 mg. of Na_2S, $9H_2O$ (240·2) were dissolved in 25 c.c. of water giving $5 \cdot 2 \times 10^{-2}$ M solution, 0·2 c.c. of which contains 2·5 mg. of sodium sulphide corresponding to 231 c.mm. of H_2S. In the first series of experiments the left-hand flask of the differential manometer received 3 c.c. of water, while the right-hand flask received 2·8 c.c. of 0·1 N HCl and 0·2 c.c. of Na_2S solution, put in a small cup provided with a platinum hook, by means of which it can be suspended from the edge of the absorption tube (Keilin, 1929). When the temperature in both flasks is equilibrated, the taps of the manometer are closed, the cup is dislodged and Na_2S, being mixed with acid, liberates the equivalent amount of H_2S, which is estimated by multiplying the manometric reading by the H_2S constant of the apparatus. The results obtained in three experiments were 221 c.mm., 224 c.mm. and 229 c.mm., which are sufficiently near the theoretical amount of 231 c.mm. The slight deficiency is partly due to a small experimental error and partly to unavoidable oxidation of H_2S at various stages of the experiment.

In other experiments 0·1 N solution of HCl was replaced by various concentrations of acid phosphate buffer and by the acid solutions of hæmoglobin

or methæmoglobin. Clear results were obtained, especially when comparatively strong concentrations of hæmoglobin or methæmoglobin and Na_2S were used. In order to liberate the total amount of H_2S in presence of a large amount of protein, it was found necessary to dissolve the latter in strong concentrations of acid buffer, which in some experiments produced a slight increase in dissociation of the H_2S-methæmoglobin compound, liberating a small excess of H_2S. Such experiments with methæmoglobin were made in the following way. The right-hand flask received 2 c.c. of $5 \cdot 2 \times 10^{-3}$ solution of methæmoglobin (estimated in terms of gm. atom of Fe per litre) mixed with 0·8 c.c. of molar solution of acid phosphate and 0·2 c.c. of $5 \cdot 2 \times 10^{-2}$ M solution of Na_2S in the cup suspended from the absorption tube. The left-hand flask received 3 c.c. of water. The experiment is then completed in the same manner as that described above. The results of this and of other similar experiments which are shown in Table IV may be summarized thus :—

Table IV.

NaH_2PO_4 M.	Hæmoglobin concentration as gm. atom of Fe per l.	Methæmoglobin concentration as gm. atom of Fe per l.	Na_2S M.	Ratio of $\frac{Na_2S}{Fe}$	H_2S mm.³.	Free H_2S% of the total.
0·13	—	—	$3 \cdot 47 \times 10^{-3}$	∞	190	82
0·26	—	—	$3 \cdot 47 \times 10^{-3}$	∞	227	98
0·46	—	—	$3 \cdot 47 \times 10^{-3}$	∞	226	98
0·26	$3 \cdot 5 \times 10^{-3}$	—	$3 \cdot 47 \times 10^{-3}$	1	207	90
0·26	$3 \cdot 5 \times 10^{-3}$	—	$3 \cdot 47 \times 10^{-3}$	1	220	95
0·26	$2 \cdot 17 \times 10^{-3}$	—	$3 \cdot 47 \times 10^{-3}$	1·6	225	97
0·13	—	$1 \cdot 73 \times 10^{-3}$	$3 \cdot 47 \times 10^{-3}$	2	114	49
0·26	—	$1 \cdot 73 \times 10^{-3}$	$3 \cdot 47 \times 10^{-3}$	2	112	48
0·26	—	$1 \cdot 7 \times 10^{-3}$	$3 \cdot 47 \times 10^{-3}$	2	136	59
0·13	—	$3 \cdot 47 \times 10^{-3}$	$3 \cdot 47 \times 10^{-3}$	1	22	9
0·13	—	$3 \cdot 47 \times 10^{-3}$	$3 \cdot 47 \times 10^{-3}$	1	26	11
0·26	—	$3 \cdot 47 \times 10^{-3}$	$3 \cdot 47 \times 10^{-3}$	1	31	13
0·26	—	$3 \cdot 7 \times 10^{-3}$	$3 \cdot 47 \times 10^{-3}$	0·9	28	12

(1) The amount of H_2S liberated when Na_2S is mixed with acid phosphate buffer can be easily estimated by means of the Barcroft differential manometer. The free H_2S estimated by this method may represent 98% of the theoretical amount of H_2S.

(2) In presence of hæmoglobin, the concentration of which in terms of iron

is approximately equal to that of Na_2S the free H_2S liberated by acid phosphate represents between 90% and 98% of the theoretical amount. This indicates that H_2S does not combine with hæmoglobin.

(3) In presence of methæmoglobin, the concentration of which in terms of Fe is only half that of Na_2S $\left(\frac{Fe}{Na_2S} = \frac{1}{2}\right)$ the amount of free H_2S liberated represents about 51% (average) of the total amount, 49% of H_2S being bound. If the concentration of methæmoglobin (as Fe) is equal to that of Na_2S $\left(\frac{Fe}{Na_2S} = 1\right)$ the amount of free H_2S is on an average 11% and of the bound H_2S, 89%.

This clearly corroborates the results previously obtained by means of spectroscopic methods, namely, that H_2S combines with methæmoglobin in the proportion of one molecule of H_2S per atom of iron of methæmoglobin.

H_2S reacts therefore with methæmoglobin in the same way as does KCN, forming a reversible compound from which hæmoglobin can be easily recovered.

The question now arises as to whether H_2S like KCN reacts with other derivatives of hæmoglobin containing trivalent iron such as hæmatin and parahæmatin. The study of these reactions, however, presents several technical difficulties and all that can be said for the present is that the absorption spectrum of alkaline hæmatin changes when a current of H_2S is passed through its solution; the band at about 617 mμ. disappears, being replaced by a wide and diffuse band at 552 mμ. That this change is not due to the reduction of hæmatin can easily be shown by passing through the solution a current of CO which does not modify its absorption spectrum. Although this observation suggests that hæmatin may react with H_2S, the product of this reaction remains still unidentified.

Summary.

(1) A compound described previously by Hoppe-Seyler, Araki, Harnack, Haurowitz and others as sulpho-hæmoglobin or sulpho-methæmoglobin, showing an absorption band at 618 mμ does not correspond to any simple combination between H_2S and hæmoglobin.

(2) This compound, from which unchanged hæmoglobin cannot be recovered, is formed only when hæmoglobin is treated with H_2S in presence of oxygen.

(3) It probably represents an irreversible ferrous derivative of hæmoglobin and some oxidation product of H_2S (such as sulphur).

(4) Acid methæmoglobin on the other hand was found to form a real compound with H_2S, showing a characteristic absorption spectrum composed of two diffuse bands: α narrow and feeble at 578 mμ, and β wider and stronger at 545 mμ.

(5) This H_2S-methæmoglobin is analogous to the compound formed between KCN and methæmoglobin, from both of which unchanged hæmoglobin can be easily recovered.

(6) Spectroscopic and manometric methods are described by means of which the formation of H_2S-methæmoglobin can be followed quantitatively.

(7) The formation of the H_2S-methæmoglobin requires one molecule of H_2S per atom of iron of methæmoglobin.

(8) The dissociation constant of H_2S-methæmoglobin was found to be approximately $1 \cdot 3 \times 10^{-5}$.

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Researches on Plant Respiration. II.—*Variations in the Respiratory Quotient during Germination of Seeds with Different Food Reserves.*

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Introduction.

Determinations of respiratory activity in plants have nearly always been made by measuring the rate of carbon dioxide evolution by the tissues. Much less frequently the rate of oxygen absorption has been used as a measure of this process. In comparatively few researches have determinations been made of both carbon dioxide evolution and oxygen absorption. Yet it is clear that a true measure of respiratory activity can only be given by one or other of these quantities alone, if they bear a fixed numerical relation to one another; where this is not so, it is obvious that at least one of these quantities cannot be taken as a measure of respiration, and it may not always be easy in such cases to decide which of them is to be regarded as giving the truer value of this process. Indeed, a determination of both carbon dioxide output and oxygen absorption by respiring tissues seems eminently desirable as providing data which should assist in the elucidation of the mechanism of the respiratory process.

In the work described in this paper, which deals with the course of respiratory activity of plants of a number of species during germination of the seeds and the early development of the seedling, we have accordingly determined not only the rate of carbon dioxide evolution, but the rate of oxygen absorption. Such determinations appear to be the more desirable as the existing information on the respiratory quotient of germinating seeds is scanty, and such data as have been recorded are highly contradictory.

For most of the information on this subject we are indebted to Bonnier and Mangin (1884), whose experiments were carried out nearly 50 years ago. Their experiments were made with *Lepidium sativum*, *Linum usitatissimum*, *Lupinus luteus*, *Vicia faba*, *Pisum sativum* and *Triticum vulgare*. Their researches indicated that generally during germination the respiratory quotient is very much lower than unity. Thus *Lepidium sativum* seedlings two days old gave a respiratory quotient of 0·35 and this had only risen to

0·41 in seedlings four days old. In only two observations was a quotient approaching unity found: in flax and in wheat at the beginning of germination. Bonnier and Mangin made some attempt at following the change in the quotient with development of the seedling in each case, though, with the one exception of flax, only 3 or 4 determinations were made with each species over a period of 2 or 3 weeks. The results with the fat-containing flax gave a quotient at the beginning of germination of 0·94, but it rapidly fell to a minimum of 0·30 by the fourth day from the beginning of germination; it then rose very slowly, reaching a value of 0·63 in a fortnight from germination and one of 0·81 after a further week. The value of the quotient showed a similar course in the starch storing wheat, though at a higher level. Here the initial value of the quotient was 1·05, a minimum of 0·61 was reached in a day, after which the value of the quotient rose to 0·86 the next day and after that slowly towards unity. A similar course in the value of the quotient is suggested by their results with other plants, to which reference will be made later, as well as with *Lepidium sativum*, where four sets of seedlings in order of age gave values of 0·55, 0·36, 0·41, 0·56. In *Pisum sativum*, seedlings of three different ages gave values of the same order, namely, 0·53, 0·65, 0·65.

Bonnier and Mangin's results are in strong contrast with the more recent determinations of the respiratory quotient of germinating seeds made by Fernandes (1923) and Frietinger (1927). The former found the respiratory quotient at 20° C. of germinating seeds of *Pisum sativum* from the first to the third hour after the commencement of water absorption to be 3·33, and during the next two hours 2·20, after which it slowly fell until after 30 hours it had reached a value of about 1·1, round about which it remained for the next four days. Frietinger obtained even higher values for germinating seeds of the same species. After seeds had swollen in water for 16 hours he obtained a value for the quotient of 4·2 which fell to about 2·8 in 7 hours and to 2·3 after a further 24 hours. Although removal of the testa brought about a lowering of the quotient the values for this were still very high, namely, 2·4 after 16 hours swelling in water and 1·4 after a further 24 hours. He also obtained values for the respiratory quotient of germinating wheat well above unity.

Of a seed such as that of *Pisum sativum*, where carbohydrate is so abundant a food reserve and fat scanty, we should not expect the low quotients obtained by Bonnier and Mangin, nor, on the other hand, is it readily understandable why, at any rate when the testas are removed, the quotient should be as high as it was found to be by Frietinger. Kidd (1915) found a value of 1·05 for

the respiratory quotient of germinating peas without testas over a 24-hour period.

Material and Methods.

We have investigated the course of respiration and variation in the respiratory quotient during germination of the seeds and development of the seedlings of ten species chosen from different families and possessing a variety of food reserves. The species are the following; the source of the seed and the variety used are shown in each case.

Gramineae	..	*Zea mais* (Sutton's Early Sugar Corn).
Polygonaceae	..	*Fagopyrum esculentum* (Sutton and Simpson).
Tropaeolaceae	..	*Tropæolum majus* (Sutton's Tall Scarlet).
Leguminosae	..	*Lupinus luteus* (Sutton's Dwarf Yellow).
		Vicia faba (Sutton's Broad Windsor).
		Lathyrus odoratus (Sutton's What Joy).
		Pisum sativum (Sutton's Pioneer).
Euphorbiaceae	..	*Ricinus communis* (Simpson's Gibsoni mirabilis).
Cucurbitaceae	..	*Cucurbita pepo* (Sutton's Long White Vegetable Marrow).
Compositae	..	*Helianthus annuus* (Sutton's Giant Yellow Sunflower).

Of these seeds, starch constitutes the principal reserve in *Zea mais, Fagopyrum esculentum, Vicia faba, Lathyrus odoratus,* and *Pisum sativum.* The last three are also rich in protein, much more so than the first two. *Tropæolum majus* and *Lupinus luteus* contain much carbohydrate in forms which are rather vaguely described as reserve cellulose, hemicellulose or amyloid. From the results of hydrolysis of these substances they appear to be condensation products of various pentose and hexose sugars. The remaining seeds, those of *Ricinus communis, Cucurbita pepo* and *Helianthus annuus,* contain large quantities of fat and at most a very small quantity of reserve carbohydrate. The species selected thus include representatives of the chief types of seeds considered from the point of view of their reserves.

The measurements of carbon dioxide evolution and oxygen consumption were made in the manner described in the preceding paper of this series; that is, for the carbon dioxide evolution the katharometer was used in conjunction with the automatic recording system, while the oxygen absorption was calculated from the change in pressure in the respiration chamber, a record of which was also obtained automatically. Both the automatic recording

devices have already been described (Leach, 1932). In every case the subject of the respiration measurements was a single seed from which the coverings (testa, and pericarp when present) were previously removed except that of *Zea mais*. In order to obtain the desirable degree of sensitivity, in the measurement of both carbon dioxide and pressure change, plant chambers of two different sizes were used, the approximate volumes of the chambers being 50 cc. and 7 cc. respectively. The apparatus was calibrated, both for carbon dioxide and change in the total quantity of gas, by the method described by one of us (Leach, 1932).

The seedlings were kept in the dark throughout the whole period of experimentation. All experiments were carried out at 25° C.

Experimental Results.

1.—*Zea mais.*

An average of 28 analyses of sugar corn published by König (1913) gives 11·40% of nitrogenous substances, 7·77% of fat and 62·85% of nitrogen-free extractives, the last, no doubt, being largely carbohydrates, and constituting the principal storage substance. It will be noted that a moderate amount of fat is present.

That both the carbohydrates and fats are used during germination of maize was indicated long ago by analyses made by Boussingault (1868). According to these analyses the starch of 22 maize seeds weighed 6·386 grams, while that of the same number of seedlings, grown in the dark for 20 days from germination, was only 0·777 grams. Fat in the seeds amounted to 0·463 grams and in the seedlings to only 0·150 grams.

The seeds of Sutton's Early Sugar Corn used in our researches commenced to germinate at once on being brought into contact with water. The respiration rate was significant during the first hour and rose regularly throughout the course of a week. A slight decline in the rate of respiration was observed after this time in one case where measurements were continued over a longer time (Experiment 113). The general course of respiration of *Zea mais* during germination is thus similar to that observed with *Lathyrus odoratus* when the seed is deprived of its testa (*cf.* Stiles and Leach, 1932 *b*, fig. 4), except that the increase in respiratory intensity is much more regular.

The respiratory quotient during this period of development does not remain constant. In five experiments where a plant chamber having a capacity of about 50 c.c. was used, the slow rate of respiration during the

first few hours rendered the results obtained for the respiratory quotient during this period liable to a considerable experimental error. We have, therefore, neglected results obtained for the respiratory quotient in these experiments during the first few hours. To obtain reliable data for this period, experiments were carried out with a small plant chamber, by the use of which the actually recorded changes are much larger and the experimental error therefore much reduced. (Experiments 122, 124.) The variations in the respiratory quotient for germinating *Zea mais* observed in all seven experiments are indicated in Table I.

Table I.—Respiratory quotient of seedlings of *Zea mais*.

Age of seedlings in hours.	Respiratory quotient.						
	Experiment number.						
	113.	114.	115.	116.	120.	122.	124.
2·3						0·95	
3·3						0·92	0·95
4·3						0·86	0·88
6·0	0·97						
7·0			0·86	0·91			0·85
10·0					0·86		
18·0	0·89		0·81	0·85			
22·0					0·86		
27·25	0·81		0·79				
31·0				0·80	0·83		
39·5		0·78		0·73			
49·5					0·73		
53·0	0·74	0·81					
59·0		0·84					
65·0		0·855					
94·5					0·92		
120·0	0·84				0·94		
143·0	0·87			1·00			
167·0	0·92						
217·0	0·94						

From the results obtained it will be observed that the respiratory quotient at the commencement of germination is in the neighbourhood of unity, but almost immediately falls and continues to do so for a considerable time, reaching a minimum of about 0·75 in about two days under the conditions of our experiments. The minimum varies in different individuals, but this is only to be expected as the quantities of the different food reserves providing the respiratory substrate are likely to vary from seed to seed. After the quotient reaches a minimum it slowly rises.

Several explanations of the course taken by the respiratory quotient during germination of maize are possible, but the most reasonable one is that the quotient at any particular time is related to the nature of the substance or substances actually used at that time. If this is so, we may suppose that at the beginning of germination some hexose sugar, present in the seed in relatively small quantity, is used, but that with the activation of lipolytic enzymes fat soon becomes used as well. With the development of the diastatic enzymes the large reserve of starch in the seed is also drawn upon. The actual quotient at any time will thus depend on the relative quantities of fat and carbohydrate used, being nearer to 0·7 or 1·0 according to which of these is most used. That the quotient may be affected by other processes is possible. The absorption of oxygen from the medium surrounding the seed for purposes not directly concerned in respiration would lead to a lowering of the ratio of carbon dioxide evolved to oxygen absorbed by the plant, while the retention in the tissues of the carbon dioxide produced in the respiratory process would have the same effect on the quotient. It may be noted that the presence of a small quantity of water in the plant chamber is necessary and that a small amount of carbon dioxide may be absorbed by this. While it is not possible to compute the magnitude of this source of error, it seems most unlikely that it is significant, having regard to the quantity of water present, the low partial pressure of carbon dioxide outside the water, and the rate at which the gas would be absorbed. When one considers the very considerable loss of fat observed by Boussingault in germinating maize, it seems most probable that the variations in the respiratory quotient are mainly related to the materials used.

It should be pointed out that the few observations of Bonnier and Mangin (1884) on wheat indicate that for this cereal the respiratory quotient exhibits in general a similar course during the germination of the grains to that which we have observed with maize.

2.—*Fagopyrum esculentum.*

The seeds of buckwheat contain much the same content of starch and nitrogen as sugar corn, but are characterized by a much lower content of fat. Analyses given by König give a content of 11·41% of nitrogenous substances, only 2·68% of fat and 58·79% of nitrogen-free extractives.

The course of respiration of two individual seeds of a sample of buckwheat obtained from Messrs. Simpson and Sons, of Birmingham, was determined in November. The seed coats were first removed and the seeds commenced to

germinate at once on being brought into contact with water in the plant chamber, and the respiration rate rose continuously for 2 to 3 days. In one experiment (117), continued for a longer period, the respiration showed a rapid falling off, after the maximum was reached, which may be related to the conditions of the experiment as suggested by our work with *Lathyrus odoratus* (1932, *b*).

In this experiment the respiratory quotient was not determined until three days from the beginning of germination, when its value was found to be 0·915. After this time it rose until 104 hours after the beginning of germination it was 0·966. In the second experiment (119) the quotient was found to be 0·925, 0·93 and 0·99 at 29·5, 36·5 and 54·5 hours from the beginning of germination. Experiments were also carried out, as for maize, to determine the quotient during the first hours of germination. For these experiments, carried out in May, a sample of seed kindly supplied by Messrs. Sutton and Sons, of Reading, was used. From these experiments (152 and 153) the respiratory quotient appears to follow a different course from that observed with maize. With these seeds the respiratory quotient a few hours after the commencement of water absorption was 0·5 or even lower, but the quotient rose continuously. In one experiment (153) terminated after 18 hours, the quotient had risen by this time to 0·83. For the second seedling (experiment 152) the quotient had reached a value of 0·86 after 19 hours from the commencement of absorption, while a subsequent measurement made two days after the commencement of germination showed that the quotient had risen to 0·96. The values obtained in the four experiments together with some isolated measurements on other individuals are summarized in Table II, and indicate clearly that in this seed the quotient rises steadily from an early stage in germination. Having regard to the fact that seedlings of the same age are not necessarily in exactly the same stage of development, the agreement between the results obtained with the different seedlings is all that would be expected.

The results obtained show that in the first hours of germination the volume of oxygen absorbed is considerably more than that of carbon dioxide evolved, while for some hours the quotient is actually lower than that to be expected if the respiratory substrate were entirely fat. Such a low value would be obtained if part of the oxygen were used in the formation from the fat of carbohydrate, part of which, at any rate, was not immediately respired. The comparatively rapid rise of the quotient towards unity suggests that this is probably what happens, for the approach of the quotient to unity after two days suggests that by this time the respiratory substrate is predominantly

carbohydrate, and that fat is no longer playing any part in determining gaseous exchange. We may suppose, that is, that the small reserve of fat in this seed is consumed at a very early stage in seedling development.

Table II.—Respiratory quotient of seedlings of *Fagopyrum esculentum*.

Age of seedlings in hours.	Respiratory quotient. Experiment number.							
	117.	119.	123.	125.	128.	133.	152.	153.
3·75							0·47	
5·25								0·50
6·25							0·71	
8·25			0·63					0·67
9·25							0·77	
11·25								0·75
12·25							0·84	
14·25				0·79	0·805			0·79
15·25							0·86	
15·8						0·80		
16·25			0·80					
17·25								0·83
18·75							0·86	
21·8						0·85		
24·5		0·925						
33·3						0·90		
36·5		0·93						
47·25							0·96	
54·5		0·99						
72·5	0·915							
104·5	0·97							

3.—*Pisum sativum.*

In the past the edible pea has been the favourite material for researches on the respiration of germinating seeds. It is, all the same, one of the least satisfactory subjects for this work on account of the excellent medium it is for the growth of fungi and bacteria. Unless the seeds are sterilized before use it is our experience that under the conditions of experiment the development of micro-organisms on the seeds becomes significant enough after a few hours to vitiate the experimental results. On the other hand, it appears likely that sterilization with mercuric chloride or other poisons may introduce other complications which may lead, and have led, to the drawing of false conclusions.

Our first experiment (130) with this species only lasted about 4 hours. Using our smallest plant chamber both the respiration rate and change in pressure could be determined with considerable accuracy from the beginning.

The respiratory quotients obtained after 1·43, 2·43 and 3·43 hours respectively from the time the seed was brought into contact with water were 1·03, 0·98 and 0·92 respectively. As with maize the quotient at the beginning of germination is thus in the neighbourhood of unity and speedily falls off. These values of the quotient were obtained before any development of micro-organisms on the seed could be detected, and we regard them as reliable determinations. As far as they go they suggest that the quotient in this species is likely to vary in the same way as that of maize.

A second experiment (146) with this species is instructive. This experiment was continued for 16 hours, by which time the infection of the seeds with micro-organisms was very definite. Owing to the high respiration rate, the respiration record was obtained for only the first twelve hours. During this time the respiration rate rose regularly but the observed respiratory quotient, after exhibiting the fall in value which we have seen characterizes the first period of germination of starch-containing seeds, began to rise after reaching a minimum and after about 11 hours had reached unity and was still rising. This early rise in the quotient appears to be associated with the attack of micro-organisms. For various reasons, this apparent rise in the quotient appears not necessarily the direct result of the respiration of the micro-organisms, but may be due to an indirect effect of the latter on the tissues of the seed or seedling.

4.—*Vicia faba.*

As is well known, the principal reserve of the seeds of the broad bean is starch. They also contain a considerable amount of protein, but very little fat. According to analyses in König nitrogenous substances constitute 25·68%, nitrogen-free extractives (principally starch) 47·29% and fat only 1·68% of the fresh weight of the seed. They thus differ from buckwheat principally in the amount of nitrogen.

Bonnier and Mangin (1884) made a few determinations of the respiratory quotient of seedling broad beans. At an early stage when the radicles were 0·4 to 0·5 cm. long, they obtained a value for the quotient of 0·87. This subsequently fell to 0·54, 0·46 and 0·37 when the radicles were respectively 4 to 5 cm., 8 cm. and 10 cm. long. They thus record a continuous fall in the quotient throughout the development of the seedling.

We carried out two experiments with this species. Respiration proceeded at a measurable rate immediately the seed began to absorb water and rose regularly until the experiment terminated. Experiment 145 was continued

for a day during which time the respiratory quotient fell regularly from 1·23 to 0·82. The course of respiration and the quotient during the earliest stage of germination are thus in general similar to those observed with other starch-containing seeds.

An experiment (150) with a second seed of this species yielded an essentially similar result for the first hours of germination, although the observed respiratory quotient at an early stage was more nearly unity, being 1·04 after 2·25 hours as compared with 1·23 at this time in the earlier experiment. Whether the smaller size of the seed in the latter experiment had anything to do with the nearer approach of the quotient to unity it is impossible to say, but it might be expected that anaerobic respiration would be more significant in a larger seed than a smaller one, so that the latter would provide a lower respiratory quotient. After about 9 hours, when the record of carbon dioxide terminated, the quotient had fallen to 0·96 as against 1·02 in experiment 145. We may say that in the broad bean the respiratory quotient in the early stages of germination is approximately unity, but is slowly falling.

Manometric observations in this experiment after 20 hours from the beginning of germination showed that at this time the respiratory quotient was well above unity. The seed on examination was found to be infected with a fungus, and we appear to have again a case in which the presence of micro-organisms brings about a high respiratory quotient.

5.—*Lathyrus odoratus.*

In the previous paper in this series (Stiles and Leach, 1932, *b*) we have already referred to the fact that in the early stages of germination of sweet pea seeds without testas the respiratory quotient is not far removed from unity.

Table III.—Respiratory quotient of seedling of *Lathyrus odoratus.*

Age of seedling in hours.... ..	2·0	3·0	4·0	6·5	10·5	25·5	52·0
Respiratory quotient	1·01	1·00	0·92	0·86	0·85	0·93	0·97

Table III summarizes data provided by an experiment with a seed of the variety "What Joy" carried out with our smallest respiration chamber which enabled more exact determination of the quotient to be made during the early stages of germination than were possible with the larger chamber used in our earlier experiments with this species. The course of respiration over a period of 52 hours is similar to that previously observed with this species. The

respiratory quotient at the beginning of germination is approximately unity, falls fairly rapidly to a minimum of about 0·85 after 10 or 11 hours, and then slowly rises towards unity. The change in the value of the quotient during germination thus resembles that of other starch-containing seeds.

6.—*Lupinus luteus.*

The seeds of *Lupinus luteus*, when mature, are devoid of starch; they contain, however, two carbohydrates, one water-soluble, the other water-insoluble. The former was first isolated by Steiger (1887) and later the name lupeose was given to it; a molecule of it on hydrolysis gives one molecule each of fructose and glucose and two molecules of galactose. It thus appears to be a tetrasaccharide and according to Tanret (1903) is impure stachyose. The second carbohydrate constituent of *Lupinus luteus* seeds is, according to Heiduschka and Tettenborn (1927), a galacto-araban trisaccharide, each molecule of which gives on hydrolysis two molecules of pentose and one of galactose.

The chief reserves of the seeds of *Lupinus luteus* are usually described as hemicelluloses. In these seeds these appear to be condensation products of galactose and pentoses, for Schultze recorded the presence in the seeds of galactan and araban, the latter constituting about one-third of the total hemicellulose present. It is not clear how far these substances are represented by the galacto-araban described by Heiduschka and Tettenborn, but it seems reasonable to suppose that the carbohydrate reserve of *Lupinus luteus* is largely, perhaps exclusively, furnished by carbohydrates built up of pentose and hexose sugars, the latter including galactose in quantity as well as glucose and fructose. As with most Leguminosæ, seeds of *Lupinus luteus* contain much protein, but little fat. According to analyses published by König (1903), the quantities of these substances are about 4·25% of fat and 38% of nitrogenous material.

Bonnier and Mangin made a few determinations of the respiratory quotient of young seedlings of *Lupinus luteus*. With seedlings possessing radicles 4 to 5 cm. long and cotyledons still closed they obtained a value for the quotient of 0·58. At a slightly later stage, when the radicles were 8 to 9 cm. long but the cotyledons still closed, the value of the quotient had fallen to 0·42. At a later stage, with cotyledons half-opened and the first foliage leaves visible, the quotient had risen to 0·72.

Seeds of *Lupinus luteus* from which the testas have been removed germinate readily and the respiration rate is measurable almost immediately after a seed

is brought into contact with water. To take a definite example from among our experiments we may cite experiment 137, in which a single seed weighing 0·0864 gm. without testa, was introduced into our smallest respiration chamber at 11.30 a.m. Over the period 12.0 noon to 1.0 p.m. the increase in carbon dioxide in the plant chamber produced a galvanometer deflexion of 3·04 cm., while during the period 3.0 to 4.0 p.m. the deflexion had risen to 7·75 cm. These movements correspond to respiration rates of 0·754 and 1·922 mg. carbon dioxide per hour per gm. of seed. In another experiment (134) the respiration rates at 1·5 and 2·5 hours from the beginning of germination were 0·880 and 1·179 mg. carbon dioxide per hour per gm. of seed.

Experiments continued for about a day show that the respiration rate rises continually and rapidly over this period, so that at the end of this time the seedling is respiring at the very considerable rate of about 3 mg. carbon dioxide per hour per gm. of seed weight.

During this period the respiratory quotient in the seeds examined was maintained between 1·0 and 0·9; actual values obtained are summarized in Table IV.

Table IV.—Respiratory quotient of seedlings of *Lupinus luteus*.

Experiment 139.		Experiment 141.		Experiment 143.		Experiment 161.	
Age of seedling in hours.	R.Q.	Age of seedling in hours.	R.Q.	Age of seedling in hours.	R.Q.	Age of seedling in hours.	R.Q.
4·67	0·90	2·67	0·94	2·25	1·04	4·0	0·95
10·67	0·91	5·17	0·89	5·25	0·91	8·0	0·89
16·17	0·93	9·67	0·92	9·75	0·91	12·0	0·96
22·67	0·92	14·67	0·95	15·25	0·96	16·0	0·97
		18·67	0·94			20·0	0·95
		72·67	0·76			24·0	0·91
						49·0	0·76

The rapid development of respiratory activity in the seed without the testa may be contrasted with that of the seed retaining its testa. In an experiment with such an intact seed the respiration rate after 140 hours was only 0·021 mg. carbon dioxide per hour per gm. of seed.

From these observations it would appear that the respiratory quotient, if it is about unity at the beginning of germination, falls rapidly to values round about 0·92, at which general level it remains for some time. Determinations with older seedlings (Experiments 141 and 161) gave lower values for the quotient suggesting that here also there is a use of fat.

7.—*Tropæolum majus.*

The so-called reserve cellulose or "amyloid" of the seeds of *Tropæolum majus* is probably similar in general to that of *Lupinus luteus*; at any rate it also appears to involve galactan in some quantity, and it is reasonable to suppose that it consists of condensation products of hexose sugars, and perhaps pentose sugars as well. The seeds of *Tropæolum majus* contain more fat than those of *Lupinus luteus*; a determination of the fat content of seeds of Sutton's Tall Scarlet "Nasturtium" kindly made for us by Mr. W. J. Rees showed that they contained 8·78% of fat, calculated on the fresh weight of the seed, corresponding to 9·66% of the dry weight.

We found the germination of seeds of *Tropæolum* without the pericarp rather variable under conditions of experiment, but experiments with two individual seeds were carried on for relatively long times, namely, 263 and 266 hours respectively. With both these seeds germination was a slow process and contrasted remarkably with the rapid germination of seeds of *Lupinus luteus* where the seed is presumed to contain reserve material of similar chemical composition. It was not until the lapse of from 3 to 4 days from the beginning of germination that the respiration rate rose above a comparatively low value, this rise corresponding to the period of obvious growth of the radicle and plumule. The further course of respiration is similar to that already recorded for the starch-containing *Zea* and *Lathyrus*.

The respiratory quotient by the time that germination is actively progressing is in the neighbourhood of 0·8 and varies for the most part between this value and 0·7 during the remainder of the period over which the experiments extended. The actual values are shown in Table V. The value of the quotient is thus what might be expected if the respiratory substrate were largely composed of fat with a certain proportion of carbohydrate. Should this be the true explanation of the observed values of the quotient it would appear that the fat is more readily mobilized by enzyme activity than the so-called "amyloid."

8.—*Ricinus communis.*

Castor oil seeds are essentially fat-containing, the average content of fat, according to the analyses published by König, amounting to 51·37% of the weight of the seed. As this contains about 6·5% of water, fat constitutes as much as 55% on the average of the dry matter of the seeds of *Ricinus*. The sugar content is small, usually about 2%, while starch may not be present at all, although Collier's analysis of Texan *Ricinus* seeds in 1879 gave as much

as 8·88% of starch. Nitrogenous substances amount on the average to about 20% of the fresh weight.

Table V.—Respiratory quotient of seedlings of *Tropæolum majus*.

Experiment 109.		Experiment 110.	
Age of seedling in hours.	R.Q.	Age of seedling in hours.	R.Q.
36·5	0·77	11·0	0·885
78·5	0·86	69·0	0·81
104·0	0·745	83·5	0·74
115·0	0·76	106·5	0·73
132·5	0·74	130·5	0·74
148·0	0·68	149·5	0·70
165·0	0·77	178·5	0·72
171·5	0·75	264·0	0·86
184·0	0·80		
200·0	0·77		
210·0	0·80		
223·0	0·825		
234·0	0·815		
254·5	0·76		

Our seeds of *Ricinus communis* var. *Gibsoni mirabilis* germinated readily without their testas, the respiration rate increasing rapidly from the beginning of germination until the termination of the experiment, which, for the longest, lasted 122 hours. A few hours from the beginning of the experiment the quotient is well below unity, *e.g.*, 0·81 after 6·5 hours in experiment 105; 0·85 after 6·75 hours in experiment 106; 0·81 after 6·75 hours in experiment 107. The quotient then falls gradually and regularly and it appeared to be still falling when our experiments were discontinued. At the ends of four experiments the values for the quotient were respectively 0·63 after 54 hours (experiment 106), 0·57 after 68 hours (experiment 105), 0·58 after 87 hours (experiment 108) and 0·495 after 121 hours (experiment 107). The change in the value of the quotient with time is shown by Table VI.

Two measurements of the respiratory quotient of *Ricinus* seeds made recently by Ermakoff and Iwanoff (1931) are in approximate agreement with our results. These authors found a quotient during the first day of germination, after a preliminary soaking in water for 23 hours of 0·8, and on the third day of 0·5.

It is to be observed that the quotient does not by any means maintain a constant level at about 0·7 as would be expected if the only process involving gaseous exchange between the seed and the surrounding atmosphere were the

oxidation of fat. In the earliest stages of germination the quotient is well above this value and sooner or later falls well below it, the fall being quite gradual. The higher initial quotient may be due to the presence of that small amount of sugar which chemical analyses have shown to be present in these seeds. Thus a seed weighing 0·15 gm. would contain about 3 mg. of sugar. With a respiratory quotient of 0·85 corresponding to a respiratory substrate consisting of half sugar and half fat, and a respiration rate of 0·4 mg. carbon dioxide per hour per gm. of seed, it would require 50 hours to use up this sugar. To take a definite example, it can be calculated that in experiment

Table VI.—Respiratory quotient of seedlings of *Ricinus communis*.

Age of seedlings in hours.	Respiratory quotient. Experiment number.			
	105.	106.	107.	108.
6·75	0·81	0·85	0·81	
18·5	0·75	0·76	0·76	0·70
28·0				0·68
31·0	0·70	0·74	0·67	
34·0				0·65
40·0				0·66
41·75		0·72		
53·5		0·63	0·66	
56·25			0·63	
59·25			0·61	
68·0	0·57			
76·0				0·63
82·0				0·59
87·0				0·58
121·0			0·495	

107, assuming that only sugar and fat are respired, and that the respiratory quotient is entirely determined by the complete oxidation of these substances, about 1·5 mg. of sugar were used by the time the quotient reached a value of 0·7. Assuming that sugar amounted to 2% of the fresh weight of this seed, the latter contained more than 3 mg. of this reserve, as its weight without testa was 0·1620 gm. This, and similar calculations for other seeds used, show that the higher quotient in the early stages of germination can be accounted for by assuming the respiration of a certain amount of sugar as well as fat. As germination proceeds, relatively more and more of the latter is used and the quotient gradually talls. What cannot be explained on these lines is the low value to which the quotient falls; and it would appear that

the oxidation of the fat is not complete or oxidation of other substances is taking place, or the carbon dioxide produced in the respiration process proper is absorbed in the seed. It may be recalled that for the fat-containing seed of flax, Bonnier and Mangin observed a quotient as low as 0·30 in one experiment, while in seedling cress (*Lepidium sativum*) the values they obtained for the respiratory quotient were about 0·4. Whether the respiratory quotients are actually as low as this we should hesitate to agree without confirmation of the results, but there seems no doubt that in fat-containing seeds the quotient may fall far below that to be expected from the simple gaseous exchange resulting from the oxidation of fat.

A possible explanation of the low quotient is suggested by a consideration of the changes which take place in the respiration of fat. These are actually hypothetical, but it is generally presumed that a transformation of fat to sugar takes place, either through hydrolysis by lipase or some other reaction, and that hexose sugar in an active form, such as furanose forms of glucose and fructose, constitutes the actual respiratory substrate (Stiles and Leach, 1932, *a*). If this is so, and if hexose accumulates, there will be a phase during germination when the observed quotient will be below that characteristic of a simple oxidation of fat.

The following considerations will make this point clear. If we assume the principal fat in the seed of *Ricinus* to be the triglyceride of ricinoleic acid, for which the formula $CH_3 . (CH_2)_5 . CHOH . CH_2CH : CH (CH_2)_7COOH$ has been suggested, the fat itself has the formula $C_{57}H_{104}O_9$. If this is used in respiration so that the whole of the fat which disappears is broken down to carbon dioxide and water, the reactions involved may be completely summed up by the equation :—

$$2C_{57}H_{104}O_9 + 157\ O_2 = 114\ CO_2 + 104\ H_2O,$$

which would mean that the respiratory quotient would be 114/157 or 0·73.

Now if part of the fat is converted to sugar the equation representing the sum of the reactions would be :—

$$2C_{57}H_{104}O_9 + 10\ H_2O + 43\ O_2 = 19\ C_6H_{12}O_6.$$

If then, of the fat which disappears part is oxidized to sugar, the oxygen absorbed for this purpose will have the effect of lowering the value of the observed quotient. If, for example, half the fat which disappears is completely oxidized to carbon dioxide and water while the other half is transformed to sugar, the apparent quotient will be 114/200 or 0·57, while if only a third is

respired and two-thirds converted to hexose, the apparent quotient will be only 0·47.

Now it appears to be a well-established fact that during the germination of fat-containing seeds carbohydrate is produced. This was observed by Fleury (1865) in *Ricinus* as well as in other fat-containing seeds, a result confirmed by Leclerc du Sablon (1893, 1895).

About the actual course taken in fat oxidation in the germinating seed and young seedling, we know practically nothing. To explain results with hemp similar to those already noted with *Ricinus* Detmer (1880) proposed the equation :—

$$C_{18}H_{34}O_2 + 27\,O = 2\,(C_6H_{10}O_5) + 6\,CO_2 + 7\,H_2O$$

to represent the formation of carbohydrate from fat. This equation, although not acceptable to-day as it stands, actually embodies the system suggested above to account for the low respiratory quotient of fat-containing seeds.

9.—*Helianthus annuus.*

Sunflower is another plant in which the reserve substances of the seed are principally fats. According to analyses collected in König, fat averages about 44·3% of the seed without pericarp, while an analysis by Frankfurt (1894) gives a value (for glycerides and fatty acids) of 55·32% of the dry weight. According to the latter authority the seeds contain 24% of protein and 3·78% of sugars, the numbers being based on the dry weight. The composition of the seeds is, therefore, in general, very similar to that of *Ricinus*.

The seeds without their pericarps germinate readily and the respiration rate rapidly rises. In experiment 98, the respiratory activity rose until a rate of 4·32 mg. carbon dioxide per hour per gm. of seed was reached after 29 hours. After this time the respiration rate fell, but at the conclusion of the experiment after a further 11 hours, it was found that the radicle had turned black and died, and to this must be attributed the fall in respiration rate. In experiment 99 the respiration rate rose to the high maximum rate of 7·90 mg. of carbon dioxide per hour per gm. of seed after which the fall was rather rapid. Experiment 100 was continued for 51 hours only, by which time the rate of respiration was 4·56 mg. of carbon dioxide per hour per gm. of seed, and was still rising. The general course of respiration is thus similar to that of other seeds already dealt with, but some divergences from a course of regular increase were observed.

In spite of the apparent similarity in the chemical composition of the seeds of *Ricinus* and *Helianthus*, the respiratory quotient varies in a different way in the two plants. Whereas in *Ricinus* the quotient falls regularly for days during the development of the seedling, in *Helianthus* the quotient at the beginning of germination is low, round about 0·7, rises to a maximum between 0·8 and 0·9 before the end of the first day, and then falls. Experiments (121 and 127), carried out with our smallest plant chamber in order to obtain more exact data during the first few hours when the respiration rate is low, confirmed some other observations. The subsequent fall in the value of the quotient after the maximum was reached continued until it was below that characterizing the oxidation of fat. In Experiment 100, at the end of the experiment, 51 hours from the beginning of germination, the value of the quotient was 0·63; in experiment 99 it reached a minimum of 0·55 after about 100 hours, and then slowly rose to 0·60 at the end of the experiment 45 hours later. Measurements made with another seedling 113 hours old (experiment 149) gave a value for the quotient in agreement with this, namely, 0·64. Our results with *Helianthus* are summarized in Table VII.

Table VII.—Respiratory quotient of seedlings of *Helianthus annuus*.

Age of seedling in hours.	Respiratory quotient.						
	Experiment number.						
	98.	99.	100.	118.	121.	127.	149.
3·5						0·67	
4·5	0·62						
5·5					0·74	0·75	
6·75		0·75	0·83			0·82	
10·0	0·75					0·87	
13·0	0·86						
16·0	0·76			0·83	0·80	0·89	
17·5	0·70						
18·75		0·85	0·89				
22·0	0·73						
27·5				0·80			
32·25		0·73					
38·25			0·72				
45·75		0·59					
51·0			0·63				
74·5		0·56					
99·25		0·55					
113·0							0·64
122·0		0·58					
145·0		0·60					

There is no obvious reason why the variations in the respiratory quotient with time should be so different in *Ricinus* and *Helianthus*. The simplest explanation is that the relative amount of fat completely oxidized to carbon dioxide and water and of fat partially oxidized to carbohydrate are different in the two seeds. The relatively smaller size of the sunflower seed with its high respiratory activity involves the more rapid exhaustion of the reserves, and this may account for the fact that the quotient does not appear to fall so low as in the seed of *Ricinus*, where, if our interpretation of the results obtained is correct, there must be a considerable accumulation of carbohydrate. Nevertheless, the quotient in *Helianthus* falls considerably below that characteristic of the complete oxidation of fat, and this suggests the accumulation of carbohydrate. This was actually shown by Frankfurt (1894) who found that whereas the fat decreased from 55·3 to 21·8% of the original weight of the seeds during twelve days of germination in the dark, sugars increased from 3·8 to 13·1%, cellulose (raw fibre) from 2·24 to 10·25% and so-called hemicelluloses from 0·0 to 3·4% of the original weight of the seeds. The more recent analyses of Miller (1910) are in agreement with these earlier findings of Frankfurt.

10.—*Cucurbita pepo.*

In the seeds of the vegetable marrow the reserve substance is also fat. Analyses of *Cucurbita* seeds by three different investigators are summarized in Table VIII.

Table VIII.—Composition of *Cucurbita* seeds.

(The numbers are percentages of the dry weight of the seed.)

	Peters (1861).	Laskovsky (1874).	Ulbricht (1886).
Ash	5·10	3·02	4·6
Fat	49·51	54·56	51·2
Protein	39·88	36·25	35·9
Cellulose	3·02	0·86	1·7
Dextrin	trace	1·02	6·6 (Dextrin and Other matter together)
Other matter	2·49	4·29	

It will be observed that these analyses agree in the large quantity of fat present as well as in the negligible amount of carbohydrate.

In all species previously examined the changes observed in the respiratory quotient are easily explicable in terms of the use of reserve material known to be present in the seeds. In *Cucurbita pepo*, however, this is not so. From the high percentage of fat present and the absence of sugar and starch, a low

quotient in the early stages of germination would be expected, more particularly so since the analyses of seedlings made by both Peters (1861) and Laskovsky (1874) show that as fat disappears during germination carbohydrate is formed. In spite of this, in seven experiments carried out by us the quotient remained above 0·8 for about 45 hours, and only after this time began to fall to values more characteristic of fat-containing seeds. The results of these experiments are summarized in Table IX.

Table IX.—Respiratory quotient of seedlings of *Cucurbita pepo*.

Age of seedling in hours.	Respiratory quotient. Experiment numbers.						
	142.	144.	148.	154.	155.	157.	160.
3·5	0·90					0·78	
4·5							0·74
5·5					0·79	0·73	
6·5			0·92	0·80			
8·0						0·75	
10·5	0·91		0·92		0·81	0·84	0·85
12·5			0·88		0·94		
14·0						0·89	
16·0	0·91		0·83				0·93
17·5		0·865			0·95		
22·5	0·93					0·92	
26·0						0·90	
27·25	0·94				0·91		
29·0						0·82	0·86
34·0							0·775
40·0	0·85				0·79		
42·0		0·64					0·82
44·5							0·80
49·0				0·78	0·74		
72·0					0·63		
91·0						0·68	

The reasons for the comparatively high values of the respiratory quotient found for very young seedlings of *Cucurbita pepo* are not obvious, and any explanation is merely a matter of conjecture. At present, therefore, we prefer simply to record the observed results and leave their explanation until further investigations have provided more information on the metabolic changes taking place during the germination phase of this species.

Discussion.

The purpose of the research described in this paper was to determine the changes in the respiratory quotient during the germination of seeds, and

early development of seedlings, containing different food reserves. Previous information on this subject is exceedingly meagre and. owing to the contradictory nature of the few available results, of doubtful reliability. The ten species now investigated, representative of a wide range of families of flowering plants, were chosen so as to include seeds with reserves of starch, hemicellulose and fat. With most of the starch seeds, whether they contain comparatively little protein as in *Zea Mais*, or much protein as in *Lathyrus odoratus*, *Vicia faba* and *Pisum sativum*, the respiratory quotient is in the neighbourhood of unity at the commencement of germination, but immediately and regularly falls to a minimum after which the quotient slowly rises towards unity. The value of this minimum, and the length of time in which it is reached after the commencement of germination, vary, with constant environmental conditions, with the species and the individual. The variations in the value of the quotient may be explained by supposing that with the first absorption of water a small quantity of hexose sugar present in the seeds provides the respiratory substrate. As water absorption continues, activation of diastatic and lipolytic enzymes takes place, and leads to the mobilization of other carbohydrates and fat, an appreciable amount of which is present even in typical starch seeds. The oxidation of this fat, whether through processes resulting finally in the production of carbon dioxide and water, or proceeding no further than the formation of sugar, brings about a fall in the respiratory quotient, and we may interpret the observed fall in the quotient to correspond with the oxidation of fat. The now old observation of the disappearance of fat in germinating maize supports this view. With the disappearance of fat, gaseous exchange becomes more and more predominantly dependent on the respiration of sugar and consequently the respiratory quotient rises towards unity.

In *Fagopyrum esculentum* the initial period of germination does not appear to be characterized by a falling respiratory quotient, and if there is such a fall it must take place too rapidly to be measured by any method at present available. In our experiments in which the first measurements were made only two or three hours after the seeds were brought into contact with water the quotient was very low, in the neighbourhood of 0·5 at this early stage in germination, and the quotient rose continuously as germination progressed until in seedlings a few days old it approximated to unity. We may suppose that the small quantity of fat in this seed is early oxidized, thus accounting for the initially low respiratory quotient and the comparatively early rise of the latter to unity.

With the two species the seeds of which contain so-called hemicellulose as

the chief reserve material, the respiratory quotient for a considerable time fluctuates about an average value. That this value is lower in *Tropæolum majus* than in *Lupinus luteus* can be related to the greater proportion of fat in the former.

The three typical fat-containing seeds examined, *Ricinus*, *Helianthus* and *Cucurbita*, exhibit among themselves marked differences in the changes of the respiratory quotient during germination. In *Ricinus* the quotient falls continuously for five days and from an early value of about 0·80 to 0·85 may reach a value below 0·5. In *Helianthus*, on the other hand, the quotient rises from an early value of about 0·7 to a maximum of 0·85 and then falls as in *Ricinus*, although it does not appear to reach the very low values observed in the latter. In each seed the changes in the quotient can be explained according to use of the food reserves known to be present in the seed. *Cucurbita*, alone of the species examined, exhibits values of the respiratory quotient during the early stages of germination which are not readily explicable if the fat in *Cucurbita* is similar in constitution to that in other fat-containing seeds, but even here the quotient, which maintains for about two days an unexpectedly high value, eventually falls to values more characteristic of seeds using fats. In all seeds it seems likely that the quotient ultimately rises as the fat becomes used and sugar replaces it as respiratory substrate.

The changes in the respiratory quotient vary from individual to individual. This is to be expected, as the amounts, both absolute and relative, of the different food reserves also vary from seed to seed. The changes are probably also related to the rate of development of the seedling which no doubt is dependent on other internal factors besides the quantities of the different food reserves. Different temperature conditions also, will no doubt influence the changes in the quotient. But in spite of these differences in detail the value of the respiratory quotient changes with age or development of the seedling in a way characteristic for different groups of species. From the data obtained in this investigation it will be seen that as regards the first few days of growth, these various types may be distinguished : (1) starch seeds of the *Zea* and Leguminosæ type (*Zea mais*, *Pisum sativum*, *Vicia faba*, *Lathyrus odoratus*) ; (2) starch seeds of the *Fagopyrum* type ; (3) "hemi-cellulose" seeds (*Lupinus luteus*, *Tropæolum majus*) ; (4), (5) and (6) fat-containing seeds of the *Ricinus*, *Helianthus* and *Cucurbita* types.

It may, however, well be that over a period of two or three weeks the behaviour of *Ricinus* would resemble that of *Zea* inasmuch as a minimum value of the quotient would be reached which would be followed by a rise

towards unity, the differences consisting in the time taken to reach the minimum and the actual value of this minimum—differences related directly to the relative proportions of carbohydrate and fat in the two types of seed.

In conclusion we should like to point out that we regard this investigation as partly of an exploratory character, having for its chief purpose the determination of the variations in behaviour presented by different types of seeds in the exchange of gases in respiration. While determinations of the respiratory quotient must form an essential part in the analysis of the processes involved in the respiratory function, for the elucidation of the problem of the respiratory mechanism and of seedling metabolism in general, determinations of the gaseous exchanges must be supplemented by chemical investigations of the material changes taking place in the developing seedling. Such data relating to the different types dealt with in the present research, will, we hope, be presented in subsequent contributions in this series of papers. Any detailed discussion of results we wish to defer until the publication of such data.

Summary.

The changes in the respiratory quotient during early development of seedlings of ten species have been determined, and the relation between the values of the quotient and the nature of the food reserve of the seedlings discussed. For each species the quotient exhibits a characteristic change in value throughout germination. In *Zea mais*, where the reserve is chiefly starch, the quotient is initially about unity, but falls regularly to a minimum of about 0·75, after which it rises slowly towards unity. *Lathyrus odoratus* and possibly *Pisum sativum* and *Vicia faba* exhibit comparable behaviour. In *Fagopyrum esculentum*, also a starch seed, the quotient at a very early stage of germination is only about 0·5, but rises regularly and comparatively rapidly towards unity. In *Lupinus luteus*, which contains much hemicellulose and a little fat, the quotient remains for nearly two days at about 0·92 and then falls; in *Tropæolum majus*, a hemicellulose seed containing a higher quantity of fat, the quotient is maintained at a lower level, round about 0·75, for 10 or 11 days. Of the three species examined with seeds containing much fat, *Ricinus communis* exhibits a respiratory quotient which falls regularly from about 0·85 to 0·5 in about 5 days, whereas in *Helianthus annuus* the quotient rises from an early value of about 0·75 to a maximum of 0·85 after which it falls regularly. In *Cucurbita pepo* the changes in the quotient are somewhat similar but at a higher level.

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A Manometric Analysis of the Metabolism in Avian Ontogenesis. III.—The Respiratory Quotient of the Yolk-sac and Allantois during the Last Two Weeks of Development.

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Introduction.

Experiments previously reported (Needham, 1932, *a*, *b*, *c*) have thrown some light on the metabolism of the chick embryo and its accessory structures by manometric determinations of respiratory quotient. These determinations, however, were confined to the blastoderm (*area opaca* and *area pellucida*) up to the 60th hour of incubation, of the embryo up to the 6th day, and of the yolk-sac between 2½ and 7 days of development. In order to complete the picture, it was necessary to carry out similar determinations on the yolk-sac during the remainder of development and on the allantois, from its first appearance before the 5th day to the time of hatching. These form the subject of the present paper.

The extra-embryonic membranes of the later stages of development are in some respects easier to deal with than those of the first week. But although they become less fragile they also become more heterogeneous, until at last it may be difficult, if not impossible, to select any portion of the yolk-sac, for example, which shall be representative of the system as a whole. This difficulty will be referred to below. On the other hand, the value of the membranes as sheets of living cells which can be studied manometrically without even the minor degree of traumatism involved in the cutting of razor-slices, should not be underestimated.

Materials and Methods.

The eggs used were all of pure-bred White Leghorn stock, from the flocks of Messrs. Chivers and the University Farm. They were incubated according to the standard conditions laid down by Murray (1925) and Needham (1926).

Most of the respiratory quotients were obtained as described in the first paper of this series (Needham, 1932, *b*) by the method of Dickens and Šimer

(1930), Ringer solution buffered with phosphate and containing 0·2% glucose, being the suspending medium. In certain of the experiments the glucose was omitted or other substances were added to the medium (see pp. 432 and 438). In all these experiments the gas phase was pure oxygen. Some experiments, however, were also carried out using the methods of Dickens and Simer (1931, *a*) for bicarbonate-Ringer and for serum, but special reference will be made to these in the proper place. Special reference will also be made to the method of preparation of the yolk-sac towards the end of development which is necessitated by the large amounts of bound carbon dioxide which it then contains. The general method of preparation is given in the previous paper.

The Normal Respiratory Quotient of the Allantois throughout Development.

The results obtained for the allantois from the 5th to the 21st day are given in Table I and fig. 1. A median ventral diverticulum of the hind-gut; it is

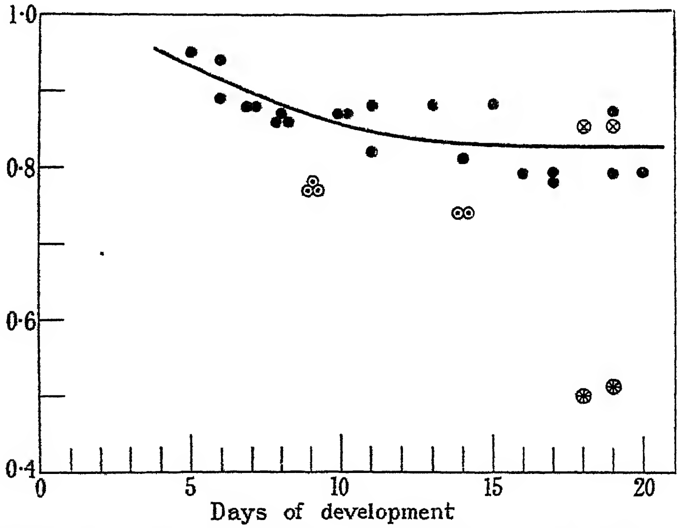

FIG. 1.—Respiratory quotient of allantois. ● Normal; ⊙ no glucose; ⊗ fluoride 20 m.mol./l.; ⊛ fluoride 50 m.mol./l.

already visible on the 3rd day, but hardly large enough for manometric work until the 5th. As the data here presented show, its respiratory quotient is at first high (0·95) but falls steadily as development proceeds to reach a mean value of approximately 0·82 by the time of hatching. It thus presents at first sight the appearance of a "mixed" quotient, protein being combusted throughout development side by side with a certain quota of carbohydrate, the latter

Table I.—Respiratory Quotient of Allantois in Ringer-phosphate-glucose medium in O_2 at 37·0°.

Experiment No.	Age in days.	Material taken. No.	Weight in mg. Total.	Weight in mg. Each.	Time of experiment. h. m.	Cub. mm. O_2.	Cub. mm. CO_2.	Cub. mm. bound CO_2.	R.Q.	Bound CO_2 in per cent. of total.
Normal.										
553	5	21	47	2	4 56	177	185	16	0·95	8·7
464	6	3	113	37	5 6	247	257	36	0·89	14·0
462	6	3	130	43	5 6	313	320	25	0·94	7·8
456	7	2	131	65	4 25	235	223	17	0·88	7·3
459	7	3	198	66	4 26	280	273	25	0·88	9·2
467	8	1	309	309	3 17	259	261	36	0·87	13·8
468	8	1	213	213	4 21	212	214	32	0·86	15·0
469	8	1	186	186	4 21	233	231	31	0·86	13·4
472	10	portion	256	—	3 51	170	164	17	0·87	10·4
473	10	,,	276	—	3 51	142	140	17	0·87	12·1
481	11	,,	217	—	6 26	205	197	30	0·82	15·2
484	11	,,	354	—	6 26	240	245	39	0·88	15·9
485	13	,,	373	—	4 36	276	274	30	0·88	11·0
501	14	,,	232	—	2 31	276	250	25	0·81	10·0
488	15	,,	500	—	1 41	326	321	34	0·88	10·6
513	16	,,	361	—	3 16	380	330	33	0·79	10·0
491	17	,,	565	—	1 51	335	314	52	0·78	16·6
493	17	,,	452	—	2 28	322	296	44	0·79	14·9
496	19	,,	639	—	3 1	354	329	21	0·87	6·4
498	19	,,	498	—	4 21	375	326	35	0·79	10·7
518	20	,,	487	—	4 36	310	275	32	0·79	11·6
Without glucose.										
476	9	1	153	—	5 10	167	148	20	0·77	13·5
477	9	1	204	—	5 10	191	166	20	0·77	12·0
478	9	1	287	—	5 10	230	202	20	0·79	9·9
506	14	portion	220 + yolk		3 26	316	270	35	0·74	12·9
508	14	,,	233 + yolk		4 21	262	314	45	0·74	14·3
Embryo, for comparison.										
556	5	4	159 + glucose		2 51	300	328	22	1·02	6·7
557	5	4	111 — glucose		2 51	191	184	15	0·89	8·2
558	5	4	171 — glucose		2 51	282	267	23	0·87	8·6
559	5	4	215 — glucose		2 51	329	314	29	0·87	9·3
Effect of fluoride										
521	18	portion	617 + 20 m.mol./l.		4 57	130	166	55	0·85	33·0
523	18	,,	542 + 50 m.mol./l.		4 57	78	91	52	0·50	57·0
526	19	,,	670 + 20 m.mol./l.		4 1	117	140	42	0·85	30·0
528	19	,,	533 + 50 m.mol./l.		4 1	65	95	62	0·51	65·0

being more proportionately at the earlier stages than at the later. On the other hand, the combustion of protein may be minimal throughout, in which case the katabolism of the later period would be mainly due to carbohydrate and fat. Decision between these possibilities cannot be made until some method has been devised for estimating the nitrogen katabolism of the isolated allantoic

membrane. It is hoped to return to this problem. Meanwhile, Borger and Peters (1933) report that the peptidase activity of the allantois on the 10th day of development is 5 times less than that of the embryo, and 60 times less than that of the yolk-sac.

It is interesting that in the earliest stages, when the allantois is growing out rapidly from the embryonic body, the respiratory quotient it gives should be close to that of the embryo itself.

It should be understood that in the present account "allantois" means "allanto-chorion," and that no distinction was made between the allantois itself, and the mesoderm of the somatopleure, with which, after the 5th day, it is fused. The only information we have on the metabolism of the somatopleure alone is an interesting unpublished experiment of Dickens and Woodger (1932) who found that the pure chorion of 4th day embryos has an extremely high anaerobic glycolysis. This is high for the allanto-chorion too, but diminishes with age.

The Effect of Altered Conditions on the Respiration of the Allantois.

In addition to the main body of the experiments, which were made in the presence of glucose (0·2% in the medium), some experiments were carried out in its absence (476–508 in Table I). The results (represented also in fig. 1) show clearly a diminution of respiratory quotient amounting to about 0·1. For purposes of comparison, similar experiments were made with embryos of five days' incubation; here also there was a diminution of about the same extent, the quotient falling from unity to 0·88.

It was previously reported (Needham, 1932, *c*, p. 126) that after inhibition of embryo glycolysis with fluoride, the residual respiration could be quantitatively accounted for by the formation of ammonia. The respiratory quotient was therefore practically unchanged. Dickens and Greville (1932) have shown that the "autoglycolysis" (the glycolysis when no substrate is added) of the chick embryo is very small, *i.e.*, $Q_M^{N_2}$ 2·7 as an average figure during the first hour, after which it quickly fell off. The autoglycolysis of the rat embryo was of the same order. It would thus appear that the 5th day chick embryo can embark on a course of protein combustion if carbohydrate is not available, either by absence from the medium, or by fluoride blockage, but that in the former condition urea is produced, in the latter ammonia. This would be understandable if an effect of the fluoride ion on one of the enzymes concerned in urea-formation could be postulated. But it might also be due to the fact that the total oxygen-consumption of the embryo is much reduced in the

presence of fluoride, and as Krebs and Henseleit (1932) and many other workers have shown, agents which diminish the oxidations in general, diminish the formation of urea, which is an endothermic reaction. As Table II shows, neither allantois nor embryo suffer a substantial diminution of respiratory rate when the medium contains no sugar.

Table II.

		Experiment No.	Cub. mm. O_2 taken up per gm. wet weight in 3 hours.
Allantois	Glucose	467	780
		468	730
		469	910
		472	565
		473	540
			—— av. 705
	No glucose	476	700
		477	640
		478	550
			—— av. 630
			Diminution = 10·6%
Embryo	Glucose	556	1890 1890
	No glucose	557	1720 ——
		558	1660
		559	1530
			—— av. 1637
			Diminution = 13·4%

It may be noted from Table I that in certain experiments, yolk was added when glucose was taken away, but without causing any greater fall in the respiratory quotient of the allantois. The only specimen of pure mammalian allantois examined by Dickens and Greville (1932) was obtained from the rabbit, in which the autoglycolysis was variable (1·0 to 12·0). The allanto-chorion of the cat and ferret, however, gave values for autoglycolysis of the same order as the chick embryo. It is probably legitimate to conclude, from all these experiments, that the chick embryo and the chick allantois have but a small supply of carbohydrate upon which they can draw in the absence of sugar in the medium. This conclusion is of some interest in view of the relatively large quantities of non-glucose, non-glycogen, carbohydrate contained in the early chick embryo (Needham, 1931).

A few experiments were tried with allantois and fluoride. As indicated in Table I and fig. 1 20 millimols sodium fluoride per litre caused an 82% inhibi-

tion of respiration but no effect on RQ, while when 50 millimols were added the respiration decreased by 87·5% and the respiratory quotient from 0·85 to 0·50. Emphasis cannot be laid on the absolute value of the latter quotient owing to the large proportion of bound CO_2 relative to the inhibited respiratory output of CO_2. It can easily be seen from these figures, however, that the sensitivity of allantoic respiration to fluoride follows a similar course to that of the yolk-sac (see Needham, 1932, *c*, fig. 8) and does not resemble that of the embryo.

The Normal Respiratory Quotient of the Yolk-sac throughout Development.

The results obtained for the normal respiratory quotient in the yolk-sac are given in Table III and fig. 2. From the latter it can be seen that the

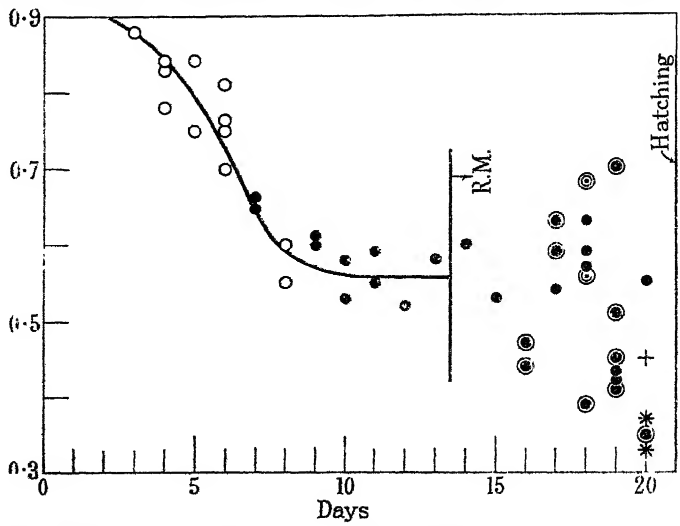

Fig. 2.—Respiratory quotient of yolk-sac. Previous paper: ○ normal. Present paper: ● normal; ⊙ no glucose; ✳ quinine; + atoxyl. If the bound CO_2 exceeds 25% of the total CO_2, the point is encircled by a ring.

values now reported fit on satisfactorily to those given in the previous paper (1932, *b*) for the first week of development. But before examining the results further, attention must be drawn to an unexpected but serious difficulty in the determination of the yolk-sac's respiratory quotient, namely, the very large amounts of bound carbon dioxide contained in it, especially during the last week of development.

Before the 14th day the bound CO_2 remains at a moderate level, 31 c.mm./gm.* After that time, however, it shows a rapid rise, reaching an

* The bound CO_2 of the allantois = 59 c.mm./gm. throughout development.

Table III.—Respiratory Quotient of Yolk-sac in Ringer-phosphate-glucose in Oxygen.

Experiment No.	Age in days.	Wet weight mg.	Time. h. m.	Temperature °C.	Cub. mm. O_2 taken up.	Cub. mm. CO_2 given off.	Cub. mm. bound CO_2.	R.Q.	Bound CO_2 in per cent. of total.
Preliminary experiments.									
184	17	326	—	38·4	—	—	103	—	—
190	19	301	5 18	38·2	282	151	34	0·42	22·5
206	19	1025	0 55	39·5	285	—	202	—	—
207	19	1112	1 2	39·5	304	—	200	—	—
208	19	821	0 55	39·5	340	—	200	—	—
213	20	263	—	39·5	—	—	65	—	—
Ordinary Method.									
591	7	701	1 35	38·5	391	309	54	0·65	17·5
593	7	761	1 35	38·5	388	322	66	0·66	20·5
561	9	491	2 6	38·5	358	238	19	0·61	8·0
563	9	646	2 6	38·5	428	297	41	0·60	13·8
566	10	464	1 51	38·5	362	227	19	0·58	8·4
568	10	331	3 1	38·5	329	195	21	0·53	10·8
573	11	663	1 11	38·5	356	229	32	0·55	14·0
571	11	595	1 2	38·5	388	248	20	0·59	8·1
536	12	561	1 41	38·5	382	208	9	0·52	4·3
540	13	429	2 41	38·5	439	260	7	0·58	2·7
Representative Method.									
576	14	410	3 25	38·5	391	253	19	0·60	7·5
581	15	386	3 16	38·5	373	240	41	0·53	17·1
586	16	585	2 11	38·5	376	270	104	0·44	38·5
588	16	633	2 11	38·5	344	247	85	0·47	34·5
386	17	352	5 16	36·5	233	140	14	0·54	10·0
312	17	310	1 36	37·0	109	101	36	0·59	35·5
313	17	213	1 36	37·0	62	64	24	0·63	37·5
391	18	294	4 1	38·0	155	110	12	0·63	10·9
392	18	310	4 1	38·0	200	131	12	0·59	9·2
393	18	358	4 1	38·0	264	164	13	0·57	8·2
251	18	202	3 17	39·5	90	70	35	0·39	50·0
381	19	353	5 15	38·0	263	159	51	0·41	32·0
382	19	381	5 15	38·0	217	145	51	0·45	35·2
548	19	448	4 36	38·5	149	169	93	0·51	55·0
545	19	466	4 46	38·5	342	177	29	0·43	16·4
550	19	520	4 36	38·5	309	338	123	0·70	36·5
376	20	314	4 32	38·0	260	138	48	0·35	34·8
377	20	297	4 32	38·0	206	158	45	0·55	28·5
388	20·75	386	—	36·5	—	—	145	—	—
Without Glucose.									
531	18	627	2 36	38·5	315	252	71	0·57	28·1
533	18	789	2 36	38·5	357	341	97	0·68	28·7
Effect of Quinine.									
396	20	416	2 15	37·5	172	69	12	0·33	17·4
397	20	383	2 15	37·5	130	59	11	0·37	19·5
Effect of Atoxyl.									
401	20	366	4 24	36·5	129	114	56	0·45	49·7

average of 206 c.mm./gm. These observations were made by what is here termed the "ordinary method," namely, the use of two separate yolk-sacs, one for the determination of bound, and the other for that of total, CO_2, or the arbitrary division of a single yolk-sac into two parts. But after the fourteenth day it was necessary to have recourse to a "representative method," *i.e.*, a method in which the tissue was divided into many different portions, and a representative piece taken from each portion to form the masses on which bound and total CO_2 were determined respectively. In this way it was hoped to avoid the one-sided effect of a local carbonate deposit, since a small piece of it would be added to both the manometers concerned. In practice the method was carried out by distributing the yolk-sac into from eight to twelve Petri dishes and then selecting pieces as nearly equal in size as possible from each dish, to form the mass of experimental material. With this method, it was possible to reduce the bound CO_2 to an average of 93 c.mm./gm., but even with its use, specimens were met with in which enormous amounts of bound CO_2 appeared, suggesting that the division of the tissue had not been sufficiently extensive. These were judged as failures of the representative method.

The importance of the bound CO_2 lies, of course, not so much in its absolute value as in the relation it bears to the CO_2 of respiration.* As will be seen from Table III the bound CO_2 in per cent. of the total CO_2 remains very low before the 14th day, always under 20%, as is also reported in the previous paper (Needham, 1932, *b*, Table VI) for the first week of development. After the 14th day, however, and in spite of the representative method, the figure may be anything up to 55%. With the allantois, on the other hand (see Table I), it never exceeds 17%.

There are thus two different sources of error in the determination of the yolk-sac's respiratory quotient, (*a*) the bound CO_2 may be unavoidably very high in per cent. of the total CO_2, (*b*) the representative method may, in a given instance, fail to ensure that the pieces of yolk-sac in the two manometers are accurately comparable as regards bound CO_2. In this connection experiments 314–316 should be noted. Here a 17th day yolk-sac, treated by the representative method, gave in three separate determinations of bound CO_2, 150, 54, and 19 c.mm./gm. Wide variation may occur in the bound CO_2 in one single yolk-sac, even after partition into, and representative selection from, six portions.

Before discussing the significance of fig. 2 an explanation for the high bound

* And since the respiratory rate of the yolk-sac falls off after the 14th day (see Needham, 1932, *a*, fig. 6), this is considerable in the last week of development.

CO_2 of the yolk-sac may be given. It has long been known that a considerable part of the calcium required for the construction of the embryonic bones is derived from the egg-shell (evidence in Needham, 1931, § 13.2). Buckner, Martin, and Peter (1925) were able to imitate the action of the chick's respiratory CO_2 by bubbling the gas through a small quantity of distilled water in an emptied egg; in this way the shell gave up $1\frac{1}{2}$ mg. of calcium as bicarbonate per hour. Calcium entering the allantoic circulation in this form, then, may be held in the form of carbonate in the yolk-sac. The calcium content of the yolk-sac itself has probably never been estimated, for when Plimmer and Lowndes use the word (1924), they no doubt imply the yolk-sac and its contents. Nevertheless, their estimations show well how the calcium in the yolk and yolk-sac together increases during the latter part of incubation, and there is a correlation between calcium-intake and the yolk-sac's bound CO_2. Possibly the deposition of calcium in the yolk-sac is a result of an intake somewhat faster than the embryo can absorb. The quantity of calcium found by Plimmer and Lowndes (1924) would much more than account for the bound CO_2 now observed, but it is not yet possible to say how much of the calcium is in the yolk-sac and how much is in the yolk.

Returning now to fig. 2 it is evident that the respiratory quotient of the yolk-sac falls from an almost carbohydrate level early in development to a very low level during the last fortnight. This low level (roughly 0·5–0·6) is far beneath the characteristic level of fat combustion (0·7). The lowest quotients of all (down to 0·35) are obtained after the 14th day, and come therefore in the period when, owing to the diminished oxygen-uptake of the tissue and the increased amounts of bound CO_2, accurate quotients are most difficult to obtain. The point at which the representative method became necessary is indicated by a vertical line on fig. 2 and owing to the great amount of scatter in the subsequent points, no continuation of the previous line is made. This scatter is somewhat reduced if the average figure for bound CO_2 is used in all cases, and the number of points for which the bound CO_2 exceeds 25% of the total CO_2 is also reduced (from 12 to 4). Since the highest points obtained during the last week of development lie under 0·7, there can be no doubt that during the last fortnight, the RQ of the yolk-sac lies very much beneath the fat level. The probable significance of this phenomenon will be discussed below.

The Effect of Altered Conditions upon the Respiration of the Yolk-sac.

Two experiments (531 and 533 in Table III) were made on yolk-sac in phosphate-Ringer without glucose. The age of the yolk-sacs used brought them

within the period of great scatter, but the absence of sugar resulted in no lowering of the respiratory quotient, rather the reverse (see fig. 2). The respiratory rate showed no diminution, but a 20% increase. These facts suggest either that the usual molecules combusted at this stage of the yolk-sac's development are not carbohydrate, or that it has ample supply of its own.

All the experiments so far described were carried out in Ringer solution buffered with phosphate. To the phosphate medium, however, it is often objected that more physiological conditions can be attained when bicarbonate is used as buffer. Since the gas phase then contains carbon dioxide, the tissue is not respiring in an environmental CO_2 tension of almost zero. And *in vivo* phosphate is quantitatively much less important than bicarbonate. The empirical evidence indicates, too, that a certain distortion of the metabolism may be feared when phosphate is used as the suspending medium. Thus it is well known that the action of cyanide in completely suppressing respiration is only exerted in bicarbonate media, Dixon and Elliott (1929); Alt (1930); Warburg (1931), and that urea-production is three or four times more intense in bicarbonate than in phosphate, Krebs and Henseleit, (1932). Again Elliott (1930), found that the velocity of oxygen-uptake by sulphydryl compounds in the presence of iron and copper was decreased 50% or more in phosphate. Dawson and Platt (1928) and Barmore and Luck (1931), on the other hand, showed that the phosphate ion exerts a specific acceleratory action on pancreatic lipase and on the oxidation of glyceraldehyde by phenol-indophenol.

In these circumstances the effect of a change of medium on the respiratory rate and the respiratory quotient could hardly be predicted, but Dickens and Simer (1931, *a*) showed for a wide range of tissues (mammalian liver, kidney, testis, cerebral cortex, chorion, retina, sarcoma) that the Q_{O_2} and the RQ are identical in phosphate and bicarbonate. More recently, Kisch (1933) has again compared the two media, and finds only slight differences between them. Mammalian liver respired to exactly the same extent in phosphate and bicarbonate; heart muscle, diaphragm muscle, and retina respired more intensely in phosphate (by 20%, 20% and 130% respectively); kidney respired less intensely in phosphate (by less than 20%). The nature of the buffer also affects to a moderate degree the use of added substrates such as pyruvate and lactate. For the *a priori* reasons given above, then, it was thought necessary to make some experiments in bicarbonate medium to test how far the very low quotients of the yolk-sac might be regarded as physiological and holding good *in vivo*.

The method of Dickens and Simer (1931, *a*) was employed, with three manometers.

The respiratory quotient is calculated from the following data :—

(1) Obtained on manometer 3 :—

H_{gas} = negative pressure in millimetres; absorption of all the CO_2 from the gas phase, the manometer containing gas, iodide, and permanganate only.

(2) Obtained in manometer 2 :—

h_{B_0} = positive pressure in millimetres; evolution of CO_2 from solution, and bound CO_2 of tissue, initially.

$B_0 = h_{B_0} . k_{CO_2}{}^2$.

H_0 = negative pressure in millimetres; absorption of all the CO_2.

$C_0 = (H_0 - H_{gas})\, k_{CO_2}{}^2$.

(3) Obtained in manometer 1 :—

h = change in reading (millimetres) during the respiration period.

h_{B_1} = positive pressure in millimetres; evolution of CO_2 from solution, and bound CO_2 of tissue, after the respiration period.

$B_1 = h_{B_1} . k_{CO_2}{}^1$.

H_1 = negative pressure in millimetres; absorption of all the CO_2.

$C_1 = (H_1 - H_{gas})\, k_{CO_2}{}^1$.

Then

$$x_b = (B_0 - B_1)$$

$$x_{CO_2} = (C_1 - C_0)$$

$$x_{O_2} = \left(h - \frac{x_{CO_2} + x_b}{k_{CO_2}{}^1}\right) k_{O_2}{}^1$$

where *k*, *k* are the constants for the various manometers and gases respectively; from which the respiratory quotient follows.

Table IV shows that when the medium is buffered with bicarbonate instead of phosphate, the respiratory quotient of the yolk-sac is no higher than before. On the contrary it is somewhat lower than the other 10th to 12th day points, but not below those obtained by the representative method slightly later in development. The effect of absence of glucose from the medium was again inappreciable. The aerobic glycolysis was very small, agreeing approximately with that found by Kumanomido (1928) for the chick embryo, by Rosenthal and Lasnitzki (1928) for rat embryo liver and kidney, and by Loeser (1926) and Bell *et al.* (1928) for placental tissue.

Table IV.—Protocols of Determinations of Respiratory Quotient of Yolk-sac in Bicarbonate-Ringer Medium. Temperature 38·8° C. All readings corrected according to thermo-barometer.

	With glucose (0·2%).				Without glucose.			
	Experiments 606 and 607.		Experiments 608 and 610.		Experiments 596 and 597.		Experiments 598 and 600.	
	mm. Clerici solution.	Cub. mm.	mm. Clerici solution.	Cub. mm.	mm. Clerici solution.	Cub. mm.	mm. Clerici solution.	Cub. mm.
H_{gas}	−101·05	—	−101·05	—	−100·50	—	−100·50	—
h_{B_0}	+ 72·4	—	+ 71·58	—	+ 74·6	—	+ 72·4	—
B_0	—	+626	—	+645	—	+645	—	+652
H_0	−181·85	—	−179·45	—	−185·3	—	−184·4	—
C_0	—	−699	—	−706	—	−734	—	−755
h	− 8·61	—	− 5·61	—	− 6·5	—	− 8·35	—
h_{B_1}	+ 61·93	—	+ 68·53	—	+ 69·8	—	+ 68·1	—
B_1	—	+538	—	+590	—	+607	—	+586
H_1	−191·00	—	−189·22	—	−190·4	—	−193·9	—
C_1	—	−782	—	−759	—	−781	—	−803
x_b	—	+ 88	—	+ 55	—	+ 38	—	+ 66
x_{CO_2}	—	+ 83	—	+ 53	—	+ 47	—	+ 49
x_{O_2}	—	−222	—	−141	—	−128	—	−158
R.Q.	—	$0·37_4$	—	$0·37_6$	—	$0·36_7$	—	$0·31_0$
Wt. of tissue taken (mg.)								
Wet	—	345	—	285	—	341	—	321
Dry	—	115	—	95	—	113	—	107
Day of development	—	12	—	12	—	10	—	10
Cub. mm. O_2/gm. wet wt./hr.	—	644	—	495	—	375	—	492
Aerobic glycolysis— $Q_M^{O_2}$	—	0·76	—	0·58	—	0·34	—	0·62

The dry weight of the yolk-sacs employed was calculated from the data of Byerly (1932). The aerobic glycolysis;

$$Q_M^{O_2} = \frac{x_b}{mt},$$

where m is the dry weight of the tissue and t the time of the experiment in hours.

Ringer solution buffered with bicarbonate differs perhaps mainly from the internal environment of the organism by reason of its lack of protein, and thus it is often held that adult serum is a better medium. The original support for this was derived from the fact that Negelein (1925), using rat embryos, found a considerable aerobic glycolysis in bicarbonate-Ringer but none (except in the early stages) when inactivated horse serum was used. Similarly, Dickens and Simer (1931, *b*) later observed an appreciable reduction of aerobic

glycolysis in various tissues (*e.g.*, testis and chorion) when serum was substituted for bicarbonate-Ringer. On the other hand Kumanomido, working with both chick and rat embryos and membranes (1928) found 50% less anaerobic glycolysis in fowl or rat serum than in bicarbonate-Ringer, and spoke of an inhibitory effect exerted by the serum. Dickens and Greville's later results (1932) for anaerobic glycolysis of rat chorion in bicarbonate-Ringer fell between those of Negelein for the same tissue in horse-serum and those of Kumanomido for the same tissue in rat serum. Nevertheless, for the *a priori* reasons mentioned, some experiments were made with the yolk-sac suspended in adult fowl serum prepared according to Okamoto (1925), but without inactivation. The respiratory quotients (shown in Table V) were higher than in bicarbonate-

Table V.—Protocols of Determinations of Respiratory Quotient of Yolk-sac in Adult Fowl Serum. Temperature 38° C. All readings corrected according to thermo-barometer, and expressed in millimetres Clerici solution or cubic millimetres gas.

Experiment	611	612	613	614	615	616
Apparatus	DG7	DG8	DG9	DG10	DG11	DG12
k_{O_2}	5·81	7·82	6·34	6·12	5·87	5·95
k_{CO_2}	6·56	8·58	7·09	6·86	6·63	6·70
H_{gas}	—	−109·0	—	−109·0	—	−109·0
h_{B_0}	—	+ 70·0	—	+ 87·0	—	+ 91·0
B_0	—	+601	—	+597	—	—
H_0	—	−181·5	—	−201	—	−200
C_0	—	−622	—	−631	—	−617
h	− 41·5	—	− 42·5	—	− 32·5	—
h_{B_1}	+ 89·5	—	+ 85·5	—	+ 89·5	—
B_1	+587	—	+606	—	+593	—
H_1	−250	—	−242	—	−247	—
C_1	−925	—	−942	—	−915	—
x_b	+ 14	—	− 9	—	+ 4	—
x_{CO_2}	+303	—	+311	—	+284	—
x_{O_2}	−521	—	−542	—	−446	—
R.Q.	$0{\cdot}58_1$	—	$0{\cdot}57_4$	—	$0{\cdot}63_8$	—
Wt. of tissue taken (mg.)—						
Wet	259	263	253	260	262	0
Dry	79	80	77	79	80	—
Day of Development	10	10	10	10	10	—
Cub. mm. O_2/gm. wet wt./hr.	668	—	712	—	565	—
Q_{O_2}	− 2·2	—	− 2·35	—	− 1·85	—
Δx (CO_2-retention)	—	+ 24	—	—	—	—
Aerobic glycolysis— $Q_M^{O_2}$	0·15	—	0·05	—	0·11	—

The dry weight of the yolk-sacs employed was calculated from the data of Byerly (1932). The aerobic glycolysis;

$$Q_M^{O_2} = \frac{x_b + \Delta x}{mt},$$

where Δx is the CO_2 retention of the serum, m the dry weight of the tissue, and t the time of the experiment in hours.

Ringer but almost exactly the same as in phosphate-Ringer, and a diminution of the aerobic glycolysis was observable (*cf.* Kumanomido, 1928).

A few experiments were made upon the influence of special agents on the respiration of late yolk-sac. Iodoacetate was found to inhibit its respiration completely (experiments 186–188 for 18th day, 193–196 for 20th day), as is shown in fig. 3, and this inhibition could not be reversed by the addition of

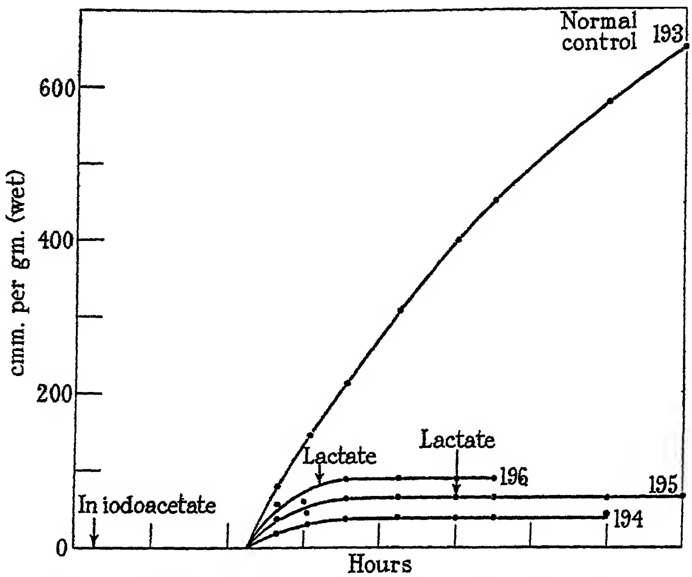

FIG. 3.—Effect of iodoacetate on late yolk-sac respiration.

193, Ringer-phosphate + 0·2% glucose.
194, 195, 196 } Ringer-phosphate + 0·2% glucose + iodoacetate N/3000.
0·2 c.c. of 2·7% sodium lactate added where indicated.

lactate.* This must mean either that iodoacetate exerts a direct effect on late stages in the oxidations or that the conversion of other substances into sugar must precede katabolic oxidation. Of these two possibilities the former is much the more probable, since Rapport (1930) has shown that no good evidence exists for the obligatory conversion of protein and fat to carbohydrate. The cent observations of Dickens (1933), Quastel (1933) and

* This agrees with the view that only tissues with a normal respiratory quotient approaching unity can have their respiration restored by lactate after iodoacetate inhibition. In the previous paper, this view was erroneously ascribed to Dickens, whose experiments concerned only the prevention of fall of respiration in inhibitants by lactate. Exceptions to the rule occur, *e.g.*, brain.

Rapkine (1933) on the reaction between the halogen-acetic acids and —SH compounds in the cell demonstrate a close connection of sulphydryl with carbohydrate breakdown (*cf.* glutathione as the co-enzyme of glyoxalase; Lohmann (1932) and Girsavicius (1933)), but it may also imply the connection originally envisaged between sulphydryl and the later oxidative stages of katabolism.

An attempt was made during the course of the work to affect the lipase of the yolk-sac by a specific agent, and following the findings summarized in Haldane's monograph (1930, pp. 160, 161) where lipases are shown to be sensitive to quinine and to atoxyl, small concentrations of these drugs were added to the suspension medium in a few respiratory quotient experiments. Quinine was used at a strength of 200 mg. per litre, and atoxyl at 5 mg. per litre. The results (Table III and fig. 2) were negative; the respiratory rate was uninfluenced and the respiratory quotient, though low, was well within the range of normal variation. If the lipolytic mechanisms of the late yolk-sac had been much affected by these agents, a marked inhibition of respiration might have ensued, but since Maeda (1923) has shown that placenta lipase, unlike all other lipases, is unaffected by quinine or atoxyl, the yolk-sac lipase probably possesses an analogous immunity.

The Significance of the Low Respiratory Quotients of the Yolk-sac.

The outstanding feature of the respiratory quotients here reported for the yolk-sac is their extreme lowness. Even if in view of the low respiratory rate and the high bound CO_2 we exclude from consideration quotients obtained after the 14th day, we have still the fact that between the 7th and the 14th day the quotient does not rise above 0·6. And, as we have seen, in no suspension medium, however physiological *a priori*, will the yolk-sac give an R.Q. lying within the limits of normal combustion. In this latter particular, it differs from the only other instance of a very low quotient in the modern literature of tissue respiration, namely, the fasting rat liver studied by Dickens and Simer (1931, *b*). Both in phosphate and bicarbonate-Ringer solutions, quotients of under 0·7 were found (phosphate 0·68–0·58; bicarbonate 0·66–0·55) but in serum the normal level for fat combustion was attained (0·73–0·71). This was not due to use of the lactate in the serum since none disappeared, and was interpreted as a damage to respiration in Ringer solutions, though the exact nature of the damage is perhaps difficult to picture. On the other hand Meyerhof and Lohmann (1926) give low figures (*e.g.*, 0·48)

for the liver of the fasting rat, even in serum. Their technique differed in certain points from that of Dickens and Simer (acidification of the serum, removal of CO_2 by evacuation).

That the low respiratory quotients cannot be ascribed to the gradual regression and eventual absorption of the yolk-sac (sometimes stated as if, even *in ovo*, it were a "dying tissue") is fairly clear from the work of Cohn and Lange (1930). These authors studied the histology and physiology of the blood vessels in the chick's yolk-sac, and failed to find there any evidence of degeneration towards the end of development. "On the day of hatching" they write "the constituent cells and fibres of the arteries of all calibres are anatomically the same as in their early development. These vessels do not die as the result of ageing; the nutrient fluid ceases to flow because of the contraction of the umbilical vessels. The blood-vessels die in complete possession of their physiological irritability and anatomical integrity, and the unaltered irritability of blood-vessels of the same calibre at all ages is consonant with their unaltered anatomical structure." Similar conclusions emerge from the work of Hammett and Zoll (1928). Biologically, the necessity for an efficient absorbing mechanism up to the last moment of pre-natal life is obvious.

Several attempts have been made to ascertain the cause of the low quotients, according to the following possibilities :—

(1) Transformation of fat to carbohydrate.
(2) Desaturation of fatty acids.
(3) Incomplete combustions, whether involving the formation of keto-bodies or not.

(1) *Transformation of Fat to Carbohydrate.*—A conversion of fat to carbohydrate by the yolk-sac in the late stages of development would be not unreasonably expected from the existing data as to the distribution and use of these two classes of foodstuffs at the time of hatching. From the work of Needham (1927) and of Vladimirov and Danilina (1930) we know that a chick embryo at the 21st day of incubation contains about 60 mg. of glycogen and about 40 mg. of other carbohydrate material. In addition some 45 mg. of total carbohydrate are found in the yolk at hatching. From Lussana's work (1906) we know that the respiratory quotient of the intact egg is 0·7 just before hatching, but rises to unity during the first two days of post-natal life. Simultaneously the liver-glycogen diminishes to zero (Shaw, 1913; and Vladimirov and Danilina, 1930). It can easily be shown by calculation that the stores of carbohydrate in the embryo and yolk at the time of hatching are insufficient

for the needs of the chick before feeding begins, if the high quotients found afterwards are really due to carbohydrate combustion. It is possible, however, that they might be interpreted as due to the evolution of retained CO_2 (*cf.* the bound CO_2 of the yolk-sac). If they are real quotients it might be reasonable to suppose that the yolk-sac forms and stores carbohydrate, though for such a formation protein might be thought a more likely source than fat.

Experiments were therefore carried out on yolk-sacs *in vitro*.

The parts of the yolk-sacs which were not used for the manometric experiments were suspended in buffered Ringer solution and maintained for the same or longer periods in the same bath as the manometers. Samples were removed at intervals and the free reducing sugar estimated according to the method of Hagedorn and Jensen (1923). When the total carbohydrate was estimated, the sample was diluted five times and HCl added to make 5%, after which the mixture was hydrolysed over a sand-bath for 4 hours and the estimation method applied.

The results are summarized in Table VI, from which it appears that an increase in free sugar occurred during the experiments, large in proportion to the sugar initially present, but extremely small in proportion to what would be needed to account for the respiratory quotient. The increase was no doubt due to the washing out of glucose from the membranes into the suspending medium, and the equalization of its concentration in the system, for when estimations of total carbohydrate were made (on yolk-sacs and medium together), the difference completely disappeared.

Table VI.—Experiments on the Formation of Carbohydrate by the Yolk-sac *in vitro*.

Experiment No.	Age in days.	R.Q. given.	Estimation.	Mg. % glucose.						% increase
				Initial.	After 2 hours.	After 4 hours.	After 6 hours.	After 7 hours.	Total increase.	
531–534	18	0·57	free	—	102	138	—	114	—	—
543–544	19	0·50	free	37	—	—	95	—	58	157
545–546	19	0·43	free	32	—	—	86	—	54	169
548–551	19	0·51	free	36	68	—	79	—	43	120
			total	470	—	—	480	—	10	2

It is concluded, then, that synthesis of carbohydrate from other materials cannot account for the low quotients of the yolk-sac.

(2) *Desaturation of Fatty Acids.*—Among the most obvious processes which might involve the use of oxygen without the corresponding evolution of CO_2

is the desaturation of fatty acids. The evidence on which desaturation has been regarded as one of the first steps, if not the first step, in the oxidation of fatty acids, has never been very extensive, but since its introduction by Leathes and Weddell (1909) it has become generally accepted (see *e.g.* the review of Bloor (1925)). New investigations which support it appear from time to time (*e.g.* Bloor and Snider (1930)). The yolk-sac might not unreasonably be regarded as the seat of active desaturating processes, since other functions normally associated with the liver are known to be undertaken by it during the chick's development (see Needham, 1931, § 8.5). It was therefore of interest to ascertain the iodine value of the fatty acids in the yolk-sac at different stages, since the desaturation (to the extent of two double bonds) of 6 molecules for every one completely combusted would be sufficient to account for the respiratory quotients observed.

The extraction and examination of the fatty acids of the yolk-sac was performed with as great precautions as possible to avoid oxidation or other changes, according to a modification of the method of Lemeland (1923). The yolk-sacs were removed from the eggs, washed in Ringer solution to remove the yolk, weighed, and ground up with anhydrous sodium sulphate (0·7 gm. to 1 gm. of tissue) to avoid the difficulties of dealing with aqueous alcoholic solutions. The powdery mass was dropped into 97% alcohol (purified from aldehydes by the method of Dunlap (1906)) and allowed to stand in the dark for two days or less, after which it was extracted in a Soxhlet apparatus for 8 hours with fresh purified alcohol. The alcoholic extracts were massed and evaporated to dryness *in vacuo*. The residue was saponified by heating for 2 hours under a reflux condenser with 25 c.c. of 2N alcoholic potash (in 97% alcohol). The mixture was then adjusted by the titration of a small aliquot part to decinormal alkalinity and an alcohol concentration of 50%, after which it was heated again for 20 minutes under the reflux condenser to ensure complete saponification. The total unsaponifiable matter was then removed by five extractions with petrol ether (boiling point 50°–60°), and the fatty acids freed by the addition of strong HCl. Five further extractions with petrol ether (boiling point below 40°) removed them from the aqueous alcoholic phase. The petrol ether was distilled off over an electric heater in a current of carbon dioxide, and the fatty acids dried at 65° for 8 hours in a vacuum oven (30 mm. Hg or less). They were then weighed and the iodine value determined by Hübl's method.

Table VII summarizes the results. The 5th and 19th days were chosen for the experiments, since as fig. 2 shows, the respiratory quotient on

the 5th day is still within the normal limits. Although a considerable increase of fatty acids and of unsaponifiable material in per cent. of the wet weight of the yolk-sac was observed, there was no appreciable change in the iodine value of the (phosphatide plus triglyceride) fatty acids. Indeed, the earlier stages gave slightly more unsaturated values, so that no explanation along these lines can be offered for the yolk-sac's respiratory quotient. The average iodine value of the yolk-sac fatty acids throughout development on the basis of the present data was 80·1, appreciably higher than the average figure of

Table VII.—Iodine Value of "intracellular" Yolk in the Yolk-sac at different stages of development.

Sample.	Age in days.	No. of yolk-sacs taken.	Material weight. gm.	Fatty acids isolated. gm.	Unsaponifiable matter isolated. gm.	% wet weight.		Weight of fatty acids sample.	Iodine value of fatty acids (gm. I/100 gm. fat).
						Fatty acids.	Unsaponifiable.		
D	5	30	8·32	0·736	0·136	8·83	1·63	0·736	89·9
E	5	28	6·25	0·620	0·104	9·90	1·67	0·620	81·2
F	5	24	5·95	0·624	0·075	10·05	1·26	0·624	79·1
A	19	4	14·75	3·336	0·221	22·60	1·50	1·112	81·1
								1·112	76·1
								1·112	74·5
								average	77·2
B	19	4	12·0	3·505	0·364	29·2	3·03	1·168	78·3
								1·220	79·1
								1·116	74·4
								average	77·2
C	20	3	4·3	1·130	0·488	26·3	11·40	1·130	76·3

73·89 obtained for the yolk fatty acids by Romanov (1932). It agrees rather with the values found by him for the embryo fatty acids (78·8 at the 16th day, rising to 90·3 at the time of hatching). This may mean that the yolk-sac takes part with the embryo in a slight progressive desaturation, but such minor processes give no solution of the present problem.

(3) *Incomplete Combustions.*—It is easy to account for the missing carbon dioxide of the yolk-sac's respiration by assuming a blockage at some relatively late stage of the oxidations, but it is not so easy to ascertain what the abnormal intermediate products are. In this connection it is important to test the

possibility that aceto-acetic or β-hydroxy-butyric acid might be accumulating in the manometer cups during the experiments. Dickens and Simer's fasting rat livers (1931, *b*), referred to above, actually showed this phenomenon, for on distilling the contents of the cups after the experiment, acetone was obtained. Some estimations were therefore made, using the van Slyke method (1917), in which, after a preliminary copper-lime precipitation, the keto-bodies are oxidized to acetone with a bichromate mixture, and the acetone estimated by titration of the insoluble mercuric sulphate compound. Thus experiment 545, which gave a respiratory quotient of 0·43, was worked up for keto-bodies with an entirely negative result, the titration figure being well within the limits of experimental error. Experiment 548 again, where an R.Q. of 0·51 was obtained, showed complete absence of keto-bodies, when the contents of the manometer cups and the main bulk of the parallel sugar-synthesis experiment were separately worked up. The outcome of these and other similar experiments was the conclusion that whatever the incomplete combustions may be, they do not lead to accumulation of the keto-bodies. This is of interest in view of the fact that although the diet of the chick embryo would be highly ketogenic for most mammals, only traces of keto-bodies can be found in the normal allantoic fluid at the end of incubation (Needham, 1933).

On the other hand some light is thrown on the situation by the fact that the respiratory quotient of protein katabolism will vary according to the incombustible end-product formed. In a former paper (Needham, 1932, *c*) it was pointed out that protein combustion ending in ammonia has a quotient of 0·95–0·97 instead of 0·81, as usually given on the assumption that urea is the end-product. Similarly, a simple calculation shows that if uric acid is the end-product (as it is in the sauropsida), the quotient will be 0·708. This lowering of the theoretical respiratory quotient, then, must be remembered in considering the extremely low quotients actually observed.

That the yolk-sac possesses *in vivo* a protein katabolism of some intensity is shown by the work of Krebs (1931) and Borger and Peters (1933) who have measured its protease and peptidase activity. This is 12 times as intense as that of the embryo on the 9th day of development.

Nevertheless, if the respiratory quotient of the yolk-sac is really about 0·56 from the 9th day onwards, a certain retention of carbon and oxygen, presumably for synthetic purposes, must be postulated. The extent to which this must occur to produce the observed effect is almost exactly 20% of the carbon dioxide eliminated. Now although this quota is significant enough in terms of the yolk-sac itself, it is not very appreciable in terms of the metabolism

of the egg as a whole, as the following figures show. Between the 10th and the 19th day one entire yolk-sac gives off about 9·85 c.c. CO_2 in 24 hours, and retains 2·4 c.c. On the 10th day the whole egg produces 50 c.c., on the 15th 200 c.c., and on the 19th day 300 c.c. The CO_2 retention of the yolk-sac therefore amounts to 2·9% of the whole CO_2 output of the egg on the 10th day, 1·6% on the 15th, and 0·5% on the 19th. This order reflects the diminishing relative ponderal importance of the yolk-sac. But the CO_2 retention may yet be a phenomenon of considerable importance in the mechanism of development. The figures in Tables III, IV and V show that it appears to be much favoured by a bicarbonate-Ringer medium.

Respiratory Metabolism in Relation to the Absorptive Work done by the Yolk-sac.

It is of interest to enquire what relations exist between the respiratory metabolism of the yolk-sac as evidenced by its respiratory quotient, and the absorptive work which it is carrying out during incubation. The first requirement here is a knowledge of the rate at which material is being absorbed from the yolk per unit weight of absorbing structure. This may be called the "absorption rate." Similarly, it is desirable to know to what extent this absorbed material is immobilized in the yolk-sac itself, either permanently as part of the edifice, or temporarily as stored substance later to be given up. The remainder of the absorbed material will pass through the vitelline vessels to the embryo and the allantois, and the rate of its passage relative to the yolk-sac weight may be called "transit rate."

In the accompanying tables the data of Byerly (1932) on the weights of yolk-sac, allantois, and embryo are employed for the purpose of such a calculation. The following symbols are used :—

y	wet weight yolk-sac (gm.)
y_i	daily increment wet weight yolk-sac (gm.)
a	wet weight allantois (gm.)
a_i	daily increment wet weight allantois (gm.)
e	wet weight embryo (gm.)
e_i	daily increment wet weight embryo (gm.)
w	$y + a + e$.
w_i	$y_i + a_i + e_i$.
c_i	daily increment combustion quota (gm.)
W_i	$w_i + c_i$.

Therefore
$$\text{Absorption rate (A.R.)} = \frac{W_i}{y} \times 100$$

and
$$\text{Transit rate (T.R.)} = \frac{a_i + e_i + c_i}{y} \times 100.$$

There are, however, certain difficulties about this calculation. The yolk-sac cannot be considered the only absorptive organ in the egg, since the allantois, especially in the later stages, probably absorbs a considerable part of the white. A.R. should also be calculated, therefore, on this assumption, and could be written

$$\frac{W_i}{y + a} \times 100.$$

Since the allantois rarely exceeds 25% of the total living matter in the egg (w) this change would not be expected to make a great deal of difference. The combustion quota (c_i), again, derived as it is from the respiration of the intact egg (data from Needham, 1931), is expressed as dry weight, and is not therefore strictly comparable with the other increments (y_i, a_i and e_i), but this is unavoidable.

The calculation shown in Table VIII starts in columns 2, 4 and 6 with the data for the weights of the constituent parts of the living matter in the egg reported by Byerly (1932), and the increments calculated from them in columns 3, 5 and 7. w and w_i are given in columns 8 and 9, c_i in column 10, and W_i in column 11. Column 12 gives the A.R., *i.e.*, the storage and combustion increment of the whole living matter in the egg in per cent. of the yolk-sac weight for the beginning of the day in question. This is given in graphical form in fig. 4. The rate of absorption is seen to be extremely high during the first five days of development, but quickly falls and oscillates around a steady level for the rest of the incubation-period, the yolk-sac then absorbing approximately its own weight each day. On the 2nd day of development, it absorbs more than three times its own weight. The allantois is brought into account in columns 13 and 14 of Table VIII, the former giving $y + a$, and the latter the modified A.R. Since the allantois does not begin its major growth-period till after the 5th day, the early part of the curve is unaffected, but in the later stages the yolk-sac and allantois together absorb at a steady rate of some 65% of their own weight each day.

If fig. 4 be compared with fig. 2 it will be seen that in the earliest stages of development, when the respiratory quotient is tending towards unity, the maximum amount of absorption is proceeding, but later on, when the absorption

Table VIII.—Calculation of "Absorption Rate" and "Transit Rate" of Yolk-sac.

(1) Day	(2) y.	(3) y_i.	(4) a.	(5) a_i.	(6) e.	(7) e_i.	(8) w.	(9) w_i.	(10) c_i.	(11) W_i.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	0·0089	0·0258	—	—	0·000786	0·00346	0·00969	0·0292	—	0·0292
2	0·0347	0·067	—	—	0·00425	0·0252	0·0389	0·0925	—	0·0925
3	0·102	0·163	—	—	0·0294	0·103	0·1314	0·2693	0·001	0·2703
4	0·265	0·212	0·00368	0·0197	0·132	0·132	0·4007	0·3637	0·003	0·3667
5	0·477	0·393	0·0234	0·0962	0·264	0·298	0·7644	0·7872	0·006	0·7932
6	0·870	0·159	0·1196	0·389	0·562	0·339	1·5516	0·8864	0·011	0·8875
7	1·029	0·215	0·508	0·544	0·901	0·587	2·438	1·346	0·020	1·366
8	1·244	0·124	1·052	0·543	1·488	0·580	3·784	1·247	0·032	1·279
9	1·368	0·422	1·595	0·243	2·068	1·100	5·031	1·765	0·045	1·810
10	1·790	0·692	1·838	—0·146	3·168	1·136	6·796	1·298	0·060	1·358
11	2·098	0·779	1·692	—0·112	4·304	1·796	8·094	2·463	0·080	1·543
12	2·877	0·293	1·580	—0·170	6·100	2·455	10·557	2·578	0·105	2·683
13	3·170	0·518	1·410	0·117	8·555	3·283	13·135	3·918	0·132	4·050
14	3·688	0·135	1·527	—0·197	11·838	2·382	17·053	2·420	0·164	2·584
15	3·823	0·191	1·330	0·184	14·320	3·250	19·473	3·625	0·198	3·823
16	4·014	0·086	1·514	0·262	17·570	4·300	23·098	4·648	0·236	4·884
17	4·100	—1·457	1·776	—0·031	21·870	2·340	27·746	0·852	0·253	1·105
18	2·643	0·170	1·745	0·081	24·210	4·060	28·598	4·311	0·259	4·570
19	2·813	—0·973	1·826	—	28·270	7·420	32·909	6·451	—	—
20	1·840		1·826		35·690		39·960			

Columns 2, 4 and 6, which form the basis of the calculation, are taken from the data of Byerly (1932).

Table VIII.—(continued).

(1) Day.	(12) $\frac{W_i}{y} \times 100.$	(13) $y + a.$	(14) $\frac{W_i}{y+a} \times 100.$	(15) $a_l + e_i + c_i.$	(16) $\frac{a_i + e_i + c_i}{y} \times 100.$	(17) $\frac{y_i}{W_i} \times 100.$	(18) $\frac{a_i}{W_i} \times 100.$
	per cent.	gm.	per cent.	gm.	per cent.	per cent.	per cent.
1	328	0·0089	328				
2	267	0·0347	267	0·0034	38	88·6	—
3	265	0·1020	265	0·0255	73	72·5	—
4	138	0·2687	137	0·1073	105	60·4	—
5	166	0·500	159	0·2527	96	57·9	5·4
6	102	0·989	90	0·4002	84	49·5	7·6
7	132	1·537	89	0·7285	83	17·9	43·9
8	103	2·296	56	1·151	111	15·7	39·9
9	131	2·963	61	1·155	92	9·7	42·5
10	76	3·628	37	1·388	101	23·4	13·5
11	74	3·790	41	0·666	37	51·0	0
12	93	4·457	60	0·764	36	50·4	0
13	128	4·580	88	2·390	83	10·9	0
14	70	5·215	50	3·532	111	12·8	2·8
15	100	5·153	74	2·449	67	5·2	0
16	122	5·528	88	3·632	95	5·0	4·8
17	27	4·876	23	4·798	119	1·8	5·4
18	173	4·388	104	1·105	27	0	0
19	—	4·639	—	4·40	167	0·4	1·8
20		—					

N.B.—Columns 17 and 18 should properly be expressed in per cent. of column 11 minus the combustions due to the respective membranes, but the error so introduced does not exceed 2%, since the katabolism of the membranes is so low in relation to the growth turnover as a whole.

rate has fallen to a steady level, the respiratory quotient has also fallen to a level state. It would be hardly safe in the present state of our knowledge to attempt to theorize about this relationship; it is sufficient to point to its existence.

In columns 17 and 18 of Table VIII y_i and a_i have been expressed in terms of W_i in order to determine the partition of the total increment of each day, and this is shown as a diagram in the upper part of fig. 4. From this it is evident that nearly the whole of the total increment before the 5th day is due to the growth of the yolk-sac. The high level of absorption (A.R.) in fig. 4

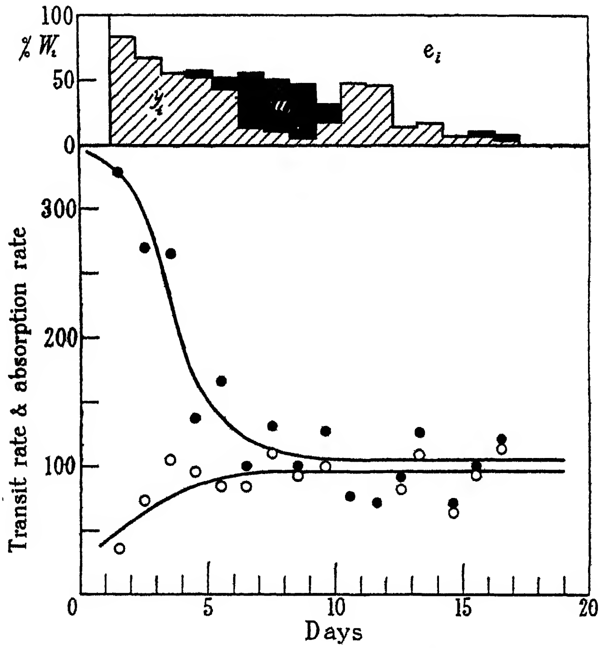

FIG. 4.—● absorption rate ; ○ transit rate.

therefore, is associated rather with the construction of the yolk-sac itself than with the function of the yolk-sac as a structure absorbing material for the benefit of the embryo. This result appears more clearly in column 16 where the transit rate is given. It shows no high level in the early stages, and demonstrates that the yolk-sac never absorbs more than its own weight of material per day *for distribution to the other structures.* The high level of absorption noted in the A.R., then, and the high respiratory quotient during the first week of development are phenomena primarily associated with the growth of the yolk-sac itself.

It will be seen that the scatter of the data in column 12 (the yolk-sac A.R.), column 14 (the yolk-sac and allantois A.R.), and column 16 (T.R.) is considerable, but this cannot be avoided where increment values are under consideration. Byerly (1932) calculated the daily increments in embryo wet weight (e_i) as a function of daily yolk-sac weight from 3 to 17 days of incubation according to the equation (expressed in the notation here used):

$$\log e_i = \mathrm{A} \log y - \mathrm{B},$$

A and B being constants. For a number of breeds, these were the same. Byerly concluded that "the efficiency of the yolk-sac is constant throughout at least the greater portion of the incubation period, and the efficiency is the same for all classes of embryos considered." This agrees with the constancy found in the transit rate.

How far the absorptive work of the yolk-sac discussed in this section is work in the thermodynamic sense, it is impossible to say. Active secretion from the yolk into the blood of the vitelline vessels may well be more important in the late stages (when the total weight of material passing is less) than in the early stages (when it is greatest). It is much to be wished that some method for investigating the true work performed by the yolk-sac may be devised.

Discussion.

Two subjects remain for comment, firstly, the general question of the validity of respiratory quotient determinations *in vitro*, and secondly, a summary of the main results of the present series of papers.

Crabtree (1932), in describing the effects of radium emanation on cell-respiration and glycolysis, points out that after some time *in vitro*, the oxygen-consumption and carbon dioxide production of a tissue may fall off unequally, so that the respiratory quotient would be merely a function of the time elapsing since the separation of the tissue from the intact organism. And for mammalian liver (though not for kidney) he shows that this difference of fall may be very considerable (*e.g.*, diminution of O_2-consumption of 50% after 9 hours, diminution of CO_2-production 70%). The relevance of this important observation is, however, lessened by the fact that the difference in rate of fall does not begin to be serious until after the fifth hour of the experiment, *i.e.*, the limit of length of any of the experiments reported in the present series of papers. Crabtree's data, plotted for the first two hours of the experiment, show practically no falling off at all (under 5%).

The question may, however, be immediately settled by plotting the respiratory quotients obtained on the blastoderm, embryo, yolk-sac, and allantois, against the time of the experiment in each case. If they give a horizontal zone, or, for those tissues whose quotient is a function of developmental age, a random distribution, then the respiratory quotient cannot have been dependent on an unequal diminution of oxygen-uptake and CO_2 production. This, in fact, occurs. The blastoderm and yolk-sac give random distributions, the embryo and allantois horizontal zones.

It was of interest to calculate the percentage negative deviation of the oxygen-uptakes recorded in the present series of papers, for some relation might exist between rate of falling off and respiratory quotient. For the purpose of comparison, the point reached half an hour after the beginning of the experiment was chosen as giving linearity, and the negative deviation after 2 hours expressed as percentage of the total oxygen-uptake reached at that time. There was no correlation between per cent. negative deviation and conditions (presence or absence of glucose, gas phase air or oxygen), age, respiratory quotient, or respiratory rate.

Ahlgren (1929) described an "apparently toxic effect" of pure oxygen in tissue respiration experiments, where the Q_{O_2} was higher in air than in oxygen, and traced it to the exhaustion of much of the substrate during the preliminary period of equilibration in the bath. No such effect was observed in the present series of experiments. The respiratory rates of the respiratory quotient experiments here reported in oxygen generally fell within the zones laid down in a previous paper for air. Only 5·8% of the experiments with yolk-sac were exceptions to this rule.

Finally, fig. 5 summarizes the main results which have emerged in the present series of papers. Before the work was begun, the respiratory quotients of the constituent portions of the living matter in the egg were quite unknown. B represents the respiratory quotients of the blastoderm unseparated into embryonic and yolk-sac parts. It is a zone of considerable scatter, probably owing to the fragility of the *area pellucida* and *area opaca*, and the relative damage to these two tissues. B_1 is the zone in which the quotients of *area opaca* alone customarily fall. The embryo is represented by four zones, E_1 giving the position of the quotients while the embryo is still sufficiently small to allow of manometric measurements without trauma, E_2 the still unknown zone of mid-development, which could only be investigated by razor slices of organs piecemeal, E_3 the final zone, deduced from the respiratory quotient of the intact egg (in which the embryo then occupies 80–90% of the living sub-

stance), and E_4, the chick after hatching. The falling allantoic zone is represented by A, and the yolk-sac by YS_1, until the respiration falls and the bound CO_2 increases so much as to make necessary the special zone of uncertainty YS_2. The quotients here are still very low, but exactly how low it is impossible to say. C represents the theoretical quotient for carbohydrate combustion, F for fat, and P_1, P_2, P_3 for protein ending in ammonia, urea, and uric acid respectively.

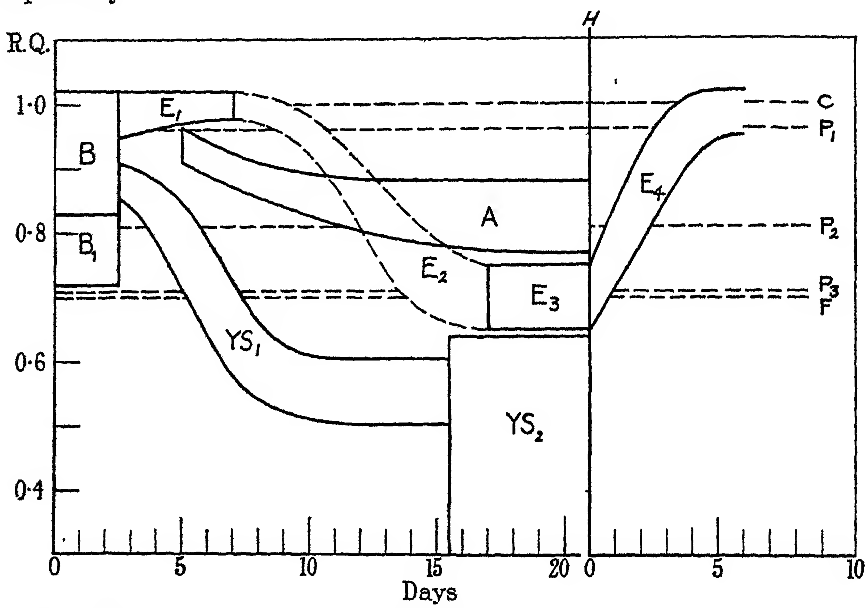

FIG. 5.—Survey of the respiratory quotients of the living components of the developing hens' egg.

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Summary.

(1) Manometric methods have been used to determine the respiratory quotient of the extra-embryonic membranes of the chick during the last two weeks of development. As suspending media, Ringer solution buffered with phosphate or bicarbonate, or adult fowl serum, were used.

(2) The respiratory quotient of the allantois falls slowly from about 0·95 on the 5th day to about 0·82 on the 20th. At the time of its origin from the

embryo, therefore, its metabolism most closely resembles that of the embryo, but later progressively departs from it.

(3) In the absence of glucose in the suspending medium the respiratory quotient of the allantois is reduced by 0·1. The embryo before the 5th day behaves similarly. A few experiments on the effect of fluoride on the allantois are described; its sensitivity resembles that of the yolk-sac rather than that of the embryo.

(4) The respiratory quotient of the yolk-sac falls from 0·9 on the 2nd day to 0·6 on the 7th after which it remains approximately constant at 0·56 until the 14th. After the 14th day an accurate quotient is difficult to obtain because (*a*) the respiratory rate is very low, and (*b*) the bound CO_2 is very high. The latter phenomenon is probably due to the localized deposition of calcium carbonate in the yolk-sac.

(5) At the mid-point of development the respiratory quotient of the yolk-sac is even lower in bicarbonate-Ringer than it is in phosphate-Ringer. In adult fowl serum it is the same. The low quotients cannot, therefore, be attributed to a damage of the tissue under *in vitro* conditions.

(6) The respiration of the yolk-sac during the last week of development is completely inhibited by iodoacetic acid in moderate doses, and the inhibition cannot be reversed by addition of lactate. Agents such as quinine and atoxyl which commonly inhibit lipases, have no effect on the respiratory quotient of the yolk-sac at this stage.

(7) The low respiratory quotients of the normal yolk-sac are shown not to be due to (*a*) the conversion of fat to carbohydrate, (*b*) the desaturation of fatty acids, (*c*) the formation of keto-bodies by incomplete combustions. They are perhaps to be explained partly by the fact that protein katabolism ending in uric acid has a respiratory quotient of 0·71 (not 0·81), and partly by a retention of some 20% of the carbon and oxygen for synthetic uses.

(8) An estimate of the absorptive work done by the yolk-sac is given. "Absorption rate" is defined as the storage and combustion increment of the whole living matter in the egg during a given period in per cent. of the yolk-sac weight at the beginning of the period in question. This is very high during the first week of development, and becomes stabilized at a lower level later. The "transit rate," however (defined as the storage and combustion increment of embryo and allantois in per cent. of the yolk-sac weight at the beginning of the period in question), shows that the high absorption rate of early development is exclusively concerned with the construction of the yolk-sac itself. The high respiratory quotient of the early stages is therefore associated with the *formation*

of the yolk-sac rather than with its *function* as a structure for the distribution of material to other structures.

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Mucus Formation in Goblet Cells.

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The formation of mucus in goblet cells and its relation to the Golgi apparatus has been studied by various workers. Nassanow (1923) showed clearly that the mucin granules in the goblet cells of Triton originated in the Golgi apparatus, and so brought secretion in these cells into line with his theory of the bound secretion. More recently Clara (1926) has shown in the goblet cells of birds that the mucin first appears in the region next to the nucleus, between it and the gland lumen. Florey (1932, *a*, *b*) has considered this more extensively in two recent papers, and for a number of mammals has shown that the mucin granules of goblet cells first form in the meshes of the Golgi network. In epithelial cells of the mouse vagina, undergoing conversion into mucous cells, he has found that the same process occurs. In a recent investigation of secretory formation in the salivary glands and pancreas it was found by the present author that in every cell type examined the young secretory granules first appeared in the basal region of the cell in relation to the mitochondria. Subsequent emigration occurred into the Golgi zone, where they underwent conversion into mature secretory granules. In the mucous cells of the salivary glands it was shown that these newly formed granules might be stained intra-

vitam by Janus green or neutral red, and that in fixed preparations they stained selectively with acid fuchsin as described by Noll (1902). In the light of this work it appeared probable that while mucin formation might occur in the Golgi zone of the goblet cells as described by these authors, the origin of the granules might lie in the basal region of the cell.

Materials and Methods.

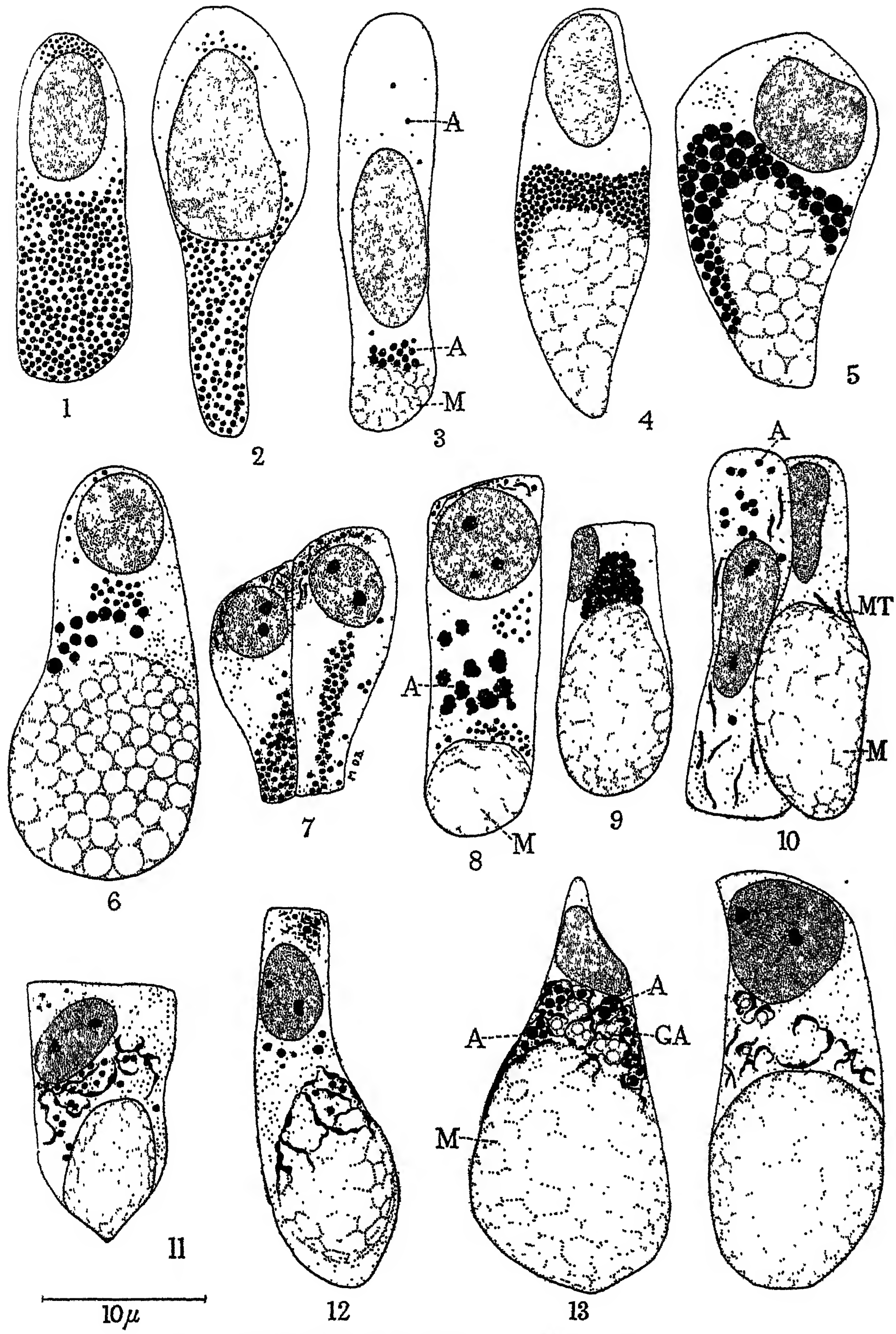
The animals used were rats 5–6 weeks old and adult mice.

Vital staining was carried out by leaving a small piece of opened colon in 1/30,000 neutral red in Ringer solution at 37° for about 15 minutes. In addition fixation for mitochondria was carried out using Regaud's fluid with post-chromatization and for the Golgi apparatus by Da Fano's and Champy's techniques.

Observations on Vitally Stained Cells.

When pieces of colon suitably stained with neutral red were examined, it was found possible to identify the various stages in goblet cell formation. Some difficulty was experienced in determining the correct orientation of cells displaying no mucin granules such as those shown in figs. 1 and 2. In these cells at or near the end of the cell there is a large group of small neutral red stained granules, and at the other pole a few small scattered neutral red stained granules. One concludes naturally that the larger end containing the greater number of granules is the end next the lumen, but this is not always certain. In later stages, fig. 3, 4, 5, and 6 when the colourless mucin granules are present the polarity is easily determined. In some cells, figs. 3 and 6, while the majority of the neutral red stained granules lie as a small cap above the unstained mucous granules, several of them may be seen at the base of the cell, A. Evidently their formation is occurring in this region. It is possible to trace a transition between these bright red granules and the less refractile colourless mucous granules so that there is little doubt that the former are young mucin granules.

In preparations fixed in Regaud's fluid and stained acid fuchsin methyl green, the process can again be easily followed. The neutral red staining granules of the living cell are now stained red with acid fuchsin, while the mucin stains a bluish green colour. In addition the mitochondria are shown. Fig. 7 shows an early stage in the goblet cell with young secretory granules at either pole, but no mucin has yet appeared in the cell. In fig. 8 mucin, M, has begun to form, and the acid fuchsin stained granules are shown clumped



A = Young secretory granules. M = mucin.
MT = mitochondria. GA = Golgi apparatus.

FIGS. 1–6.—Fresh goblet cells of colon in various stages of formation stained supra-vitally by neutral red, 1/30,000. Figs. 1–5 are from 5 weeks old rats and fig. 6 is from an adult mouse. FIGS. 7–10.—Goblet cells of colon of young rat showing as before mucin formation. Regaud's fixation. Acid fuchsin methyl green. FIG. 11–14.—Goblet cells of colon of young rat showing relation of Golgi apparatus to newly formed mucin granules. Champy's fixation with post-osmication. Counter-stained acid fuchsin methyl green.

together in little groups between it and the nucleus. This would correspond to the stage shown in figs. 4 or 5, the granules having a tendency to be clumped ino groups by the fixative. Careful focussing enables one, however, to distinguish the individual granules. Later stages are shown in figs. 9 and 10. The most usual is that in fig. 9, when the acid fuchsin granules, A, sit as a small cap on the upper pole of the mucin mass. Fig. 10 shows a fully formed goblet cell, with no fuchsinophil granules while on its left is an immature cell showing a few young granules at the periphery and mitochondrial rods MT also shown. These latter in any of the cells studied have been disposed either as long filaments in the length of the cell or as short rods parallel to the base.

The relationship of the Golgi apparatus to the mucin secretion is shown in figs. 11–14. These sections after light osmic impregnation and bleaching in hydrogen peroxide were stained with acid fuchsin methyl green. In thin sections it is possible to distinguish both the acid fuchsin stained granules, and the first mucin granules stained with methyl green as in the figures. The Golgi apparatus then as in fig. 11 is found to lie between the nucleus and the mucin, M—the fuchsinophil granules lying in its meshes. Later as the cell fills it comes to embrace the periphery of the mucin as in figs. 12 and 13, some of the fuchsinophil granules lying outside it. Stages as in fig. 13 between the red fuchsinophil granules and the green mucin granules may be distinguished, the granules in such cases lying in the network and being coloured red or green. Later as in fig. 14 the Golgi apparatus may lie outside the mucin mass, a few mucin granules being found in it.

It is in this way possible to correlate the secretory cycle in goblet cells with that found in other gland cells salivary glands and pancreas. The early secretory granules form at the periphery of the cell in the mitochondrial zone, and move into the Golgi area where they undergo transformation into mature mucin granules. The process is particularly easy to follow in this case, and occurs actually in the threads of the network. Thus while the origin of stainable mucin is in the Golgi network as maintained by Nassanow and Florey, the primary origin of the secretory granules is outside it. The whole process is in every way similar to that which occurs in the mucous salivary glands.

Summary.

(1) The origin of secretory granules in goblet cells occurs at the cell base, probably in relation to the mitochondria.

(2) Emigration into the Golgi area occurs, where the transformation into stainable mucin takes place.

(3) The whole process is in every way similar to that occurring in other gland cells particularly those of the mucous salivary glands.

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Investigations of the Mechanism of the Transmission of Plant Viruses by Insect Vectors.—I.

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[Plate 12.]

The studies here described aim at the elucidation of the action of a plant virus within the insect that is its specific vector. It is widely held that insect transmission is not normally a mechanical process; but of the nature of the biological relation, into which virus and insect are supposed to enter, little is definitely known. By the use of the method of mechanical inoculation of the virus into the insect I have obtained certain direct evidence bearing upon this problem.

The virus studied is that which causes streak disease in the maize plant. A single strain has been used, maintained in the course of my experiments by repeated transfers to maize seedlings in the greenhouse. Conceivably this strain is a complex of viruses, but if so it has shown no sign of splitting into its components during the period of the work now described.

The vectors of the streak virus are the leafhoppers, *Cicadulina mbila* Naude and *Cicadulina zeae* China. The process of transmission has been the subject of considerable past studies (Storey, 1925, 1928, 1932, *a*; Storey and McClean, 1930), and recently I have shown that within the species *C. mbila* there occur races, to which I have applied the terms *active* and *inactive*, able and unable

respectively to transmit the virus in the normal process of feeding* (Storey, 1932, *b*). Similar races probably exist in the species *C. zeae*, but inactive races have alone been available for use in these investigations. The several races have been bred through many generations, and frequent sampling tests, performed during the course of this work, have shown that purity has been maintained, except possibly in the instance of one active race.

For certain secondary studies I have used *Peregrinus maidis* Ashmead, the vector of a virus recently found in East Africa and believed to be identical with that causing the stripe disease of maize in Cuba (Stahl, 1927), and *Aphis maidis* Fitch, the vector of the common grass or sugar cane mosaic disease (Brandes, 1920).

Methods.

My method of inoculating the virus-bearing fluid into leafhoppers, a process which has apparently not hitherto been used in virus studies, is now described.

Two needles are required; one, the "holding needle" is a blunt-pointed steel needle with one flat side; the other, the "inoculating needle" is a stainless steel entomological pin (Kreye's "Insektennadeln aus rostfreiem Kruppstahl," No. 5) ground on an oilstone to a finely tapering point. For certain purposes the puncture in the insect has been made with a finely pointed steel sewing needle, which is more robust than the entomological pin but resistant to wetting and so less suitable for the transfer of inocula. The needles are sterilized before use by immersion in boiling water. Upon the stage of a binocular dissecting microscope is placed a movable stage consisting of a sheet of composition cork material, which is soft enough not to damage the delicate point of the inoculating needle. This stage is sterilized by burning its surface slightly in a flame.

For inoculation the insect is anæsthetized with chloroform and laid on its back on the stage. In the normal inoculation, which has been used throughout most of these studies, the insect is steadied by the holding needle and the inoculating needle, bearing the inoculum, is thrust into the middle of the abdomen, the point passing usually just through to the opposite side, fig. 1, Plate 12.

It should be noted that the two species of *Cicadulina* are small insects of an overall body length of 2·5–3 mm., and in the early work no attempt was made

* The assumption that the transmission process occurs during the *feeding* of the insect has been generally made. Its truth can hardly be doubted, although I know of no evidence that goes further than proving that *contact* between the insect and the plant is necessary. In this paper, however, I offer evidence showing that a period of contact with a diseased plant results in the appearance of the virus in the intestine of the insect.

to direct the inoculating needle into any particular internal organ or to decide at the time of the inoculation whether any organ had been penetrated. With the normal puncture the inoculum was certainly introduced into the blood and possibly into some organ of the abdomen. For certain purposes I have tried to avoid all organs by making a shallow puncture in the abdomen, the point only of the needle being introduced in a sloping direction just under the skin. This object has been more certainly attained by making the puncture in the femur of a hind leg. On the other hand in certain experiments I have aimed at the penetration of organs in the abdomen by making a deep puncture, the point of the needle passing right through the abdomen, fig. 2, Plate 12. I shall produce experimental evidence to support the view that in deep inoculation the needle at least occasionally may penetrate the intestine.

In certain instances, *e.g.*, the inoculation of fæces or blood, it has been possible to transfer a minute drop of fluid on the point of the inoculating needle. Usually, however, the amount of inoculum introduced has been the film adhering to the needle after dipping in the fluid. In order to increase the dose of inoculum I used in some experiments a needle that was encircled close to its point by a minute spiral of fine brass wire, fig. 3, Plate 12. This "reservoir needle" was used in the following manner: the point of the needle having been introduced into the abdomen of the insect, the fluid was drawn down from the reservoir to the puncture with the point of the holding needle. It was possible to see that a quantity of the fluid passed into the puncture. The quantity, however, was not measurable; I refer to this inoculation as "heavy" as compared with the "light" inoculation by the normal method. A refinement of the technique is the substitution for the inoculating needle of a capillary glass pipette, fig. 4, Plate 12. This pipette, made by drawing out a fine glass tube in a micro-burner, is sealed (with De Khotinsky cement) to the end of a standard hypodermic needle. The use of a Burroughs Wellcome "Agla" micrometer syringe allows of the ejection of an approximately known volume of the inoculating fluid. There are, however, a number of practical difficulties with this method and I have used it only to a limited extent.

It is surprising that the very severe wounding involved in these methods of inoculation—the severity of which may be judged by the photographs in figs. 1, 2, 4, Plate 12—has not usually proved fatal to the insects concerned. The wound in the exoskeleton is rapidly sealed by coagulation of the blood fluid. On recovering from the anæsthetic the insects usually feed at once. In a large number of experiments with *C. mbila* employing the normal puncture, a mean of 90% of the inoculated insects survived 2 days and 60% survived

a week. In many instances inoculated insects survived for 5 weeks or more. Insects inoculated by a deep puncture and by pipette showed a somewhat lower survival rate, while those inoculated with a shallow puncture showed a higher one. Even nymphs successfully underwent the change of skin within a few days of inoculation. I have raised small families of *C. mbila* from parents that, punctured while still nymphs, subsequently paired as adults and laid eggs.

The inoculation process has involved the introduction into the body cavity of various alien fluids, seldom sterile and usually not specially adjusted to be isotonic with the insect's blood. Particularly is this true of inoculations by pipette, where a relatively large volume of fluid injected has often caused the whole insect to be extremely distended. Nevertheless, no wide deviation from the usual survival rate has been noted, except in one of several experiments in the inoculation of voided fæces by needle.

An essential part of every experiment has been the test of the insects for the power to cause infections. My method for testing *C. mbila,* confined singly in a small cage clipped on to a leaf of a maize seedling, has been described elsewhere (Storey, 1928). Long experience of this technique has caused me to place great confidence in it as a decisive test whether the insect is *infective* or *non-infective.** The insects have been retained on the test seedlings usually for 7 days. This period is amply long enough to ensure infection of the test plants by normally infective leafhoppers. Recent evidence shows, however, that on rare occasions a hopper, made infective by inoculation, may fail to infect a plant within this time. However, the errors introduced into my results from this cause can have been only slight. For tests of groups of insects I have used an enlarged form of the clip-on cage, in which the leaf of the test plant is pressed against the open end of a glass tube of 15 mm. bore. It is convenient to seal on to this end of the tube a piece of "bolting-silk," which prevents the accidental escape of the insects during manipulation but does not hinder their feeding on a leaf pressed into contact with the silk. The first symptoms of streak disease have usually appeared within 3-6 days, but the test plants have been retained under observation generally for 20 days. All tests have been performed in a gauze-protected glasshouse, where control seedlings have been maintained alongside the test seedlings. In the course

* I use these terms in preference to, but in the same sense as, the "viruliferous" and "non-viruliferous" of an earlier paper (Storey, 1932, *b*). An insect is "infective" if it is actually carrying the virus in such a manner that it inoculates that virus into a plant upon which it feeds.

of this work only one control plant became diseased and this infection was clearly associated with an escaped leafhopper captured on a neighbouring plant.

The preceding methods have been applied to the inoculation of the appropriate viruses into *Peregrinus maidis* and *Aphis maidis*. A limited series of experiments, however, gave entirely negative results.

The Inoculation of Insect Vectors.

In the course of later sections of this paper many experiments in the inoculation of active races of *C. mbila* with a variety of inocula will be described in detail. At this stage it will be sufficient to summarize results that demonstrate the possibility of this process.

In nine separate experiments, in which undiluted fresh juice of streak-diseased maize seedlings was inoculated by a normal puncture into the abdomens of 150 active individuals of *C. mbila,* a total of 106 became infective. In one experiment all of 15 insects inoculated became infective; otherwise a proportion only of those inoculated became infective. The mean percentage of successful inoculations in the nine experiments was $73 \pm 6 \cdot 6$. The successful experiments included both sexes of adults and also nymphs.

In the preceding experiments the exact site of deposition of the inoculum was uncertain; further experiments showed, however, that the inoculation might be effective if the virus was introduced into the blood alone. A heavy inoculation into a shallow puncture of the abdomen made 13 out of 18 leafhoppers infective. A heavy inoculation into a puncture of the femur—certainly avoiding all internal organs—gave 18 infective hoppers out of 28.

The inoculum used was undiluted fresh juice pressed from crushed young diseased maize seedlings grown in the greenhouse. Experiments showed that this fluid, stored in the dark at 23° C., contained inoculable virus up to the fourth day after preparation, but not on the eighth day. Fresh juice was effective by heavy inoculation when diluted with distilled water by 10^{-2} and rarely by 10^{-3}. Clarification of the juice by passage through paper pulp often rendered it ineffective, even when inoculated in relatively large quantities by pipette. It may be noted, however, that the virus may usually be recovered from such clarified juice by feeding it to leafhoppers through a membrane (Storey, 1932, *a*).

A study of the transmission by inoculated insects revealed a difference in their behaviour from that of insects made infective by feeding on a source of the virus. The ultimate effect of infection by either class of vector was the

same, but, following infection by an inoculated insect, the disease usually developed in the plant more slowly. The inoculated insect caused infections more irregularly in short periods of exposure. Ultimately the inoculated insect became non-infective, whereas the insect that had taken up the virus by mouth usually continued to cause infections up to the time of its death. Detailed studies of these features of the transmission process will be described in later communications.

One further method of the introduction of the streak virus into *C. mbila* may be mentioned here. If the abdomen of the insect be gently pressed, a drop of fluid appears at the anus. On releasing the pressure the drop is drawn back into the rectum. By bringing into contact with the anus a reservoir needle bearing the juice of streaked maize and then releasing the pressure, I have supposed that some of the virus-bearing fluid would be drawn into the rectum. This experiment has been done three times, but the 77 active hoppers so inoculated all remained non-infective.

The Virus in the Vector Insect.

By the inoculation into non-infective active *C. mbila* of fluids obtained from other infective *C. mbila* I have tried to follow the behaviour of the streak virus in this insect. The infective insects, unless otherwise stated, had obtained the virus by feeding for several days upon diseased maize. In order to obtain infective insects that had not recently taken up the virus in feeding, the insects were caged on single leaves of an immune species of plant (*e.g.*, a suitable variety of sugar cane) or of a healthy maize seedling. Although the latter will be infected as a result of the insects' feeding, I have shown that the virus is only rarely picked up from the part of a plant through which it receives the virus (Storey, 1928). As an additional precaution the insects were transferred to a fresh leaf every second day.

The inoculation of fæces voided by infective hoppers during feeding on a diseased plant, slightly diluted with sterile distilled water, gave negative results when inoculated into a total of 44 insects in two separate experiments. Similar negative results were obtained by feeding* voided fæces, diluted with 20% sucrose solution, to active hoppers. This result confirms that of Severin (1931) with *Eutettix tenellus* Baker and the virus of curly-top of sugar beet If, however, the abdomen of an anæsthetized leafhopper be gently pressed with a needle a drop of clear fluid will usually appear on the anus. This

* By the method described in an earlier paper (Storey, 1932, *a*).

drop may be picked up with a second needle and inoculated into a non-infective hopper. The results of such experiments (Table I, experiments 1 and 2) show that this fluid contains the virus, if the hopper from which it is taken has recently fed on diseased maize. The low proportion of successes obtained by this inoculation suggests that the virus content of the fluid is small. If the infective hopper has recently fed only on healthy plants the fluid exuded usually contains no virus (Table I, experiments Nos. 3, 4, 5). In one of these experiments the fluid from infective hoppers, fed during the preceding 6 days only on healthy maize, gave a single infection out of 33. Where the time of feeding on healthy plants was increased to 10 and 20 days, no infections resulted.

Table I.—The inoculation of the rectal contents of infective active *C. mbila* into active *C. mbila*.

Experiment number.	Inoculum. History of infective hoppers before inoculation.		Test insects. Number inoculated.	Number infective.
		days		
1	On diseased maize	>2	25	7
2	,,	>2	26	2
3	On healthy maize	6	33	1
4	,,	20	26	Nil
5	On healthy sugar cane, Co 205	10	19	Nil

I believe that the drop of fluid exuded from the anus is actually the contents of the rectum, and not blood, such as might be expected to exude had the wall of the rectum been ruptured by the pressure exerted. If the pressure on the abdomen be heavy, however, it is possible to rupture the rectum; the sudden appearance of a large volume of blood—often bearing globules of fat—is characteristic and easily recognized. Furthermore, whereas the supposed contents of the rectum may in some circumstances, as I have shown above, contain no virus, the evidence given in the next paragraphs shows that in those same circumstances the blood will contain the virus.

Inoculation with the general body contents of infective hoppers gave positive results. To obtain the inoculum the anæsthetized insect was divided at the junction of the thorax and abdomen with a heated needle; the mixed contents of each half were then picked up by needle. The experiment was successful whether the contents of the abdomen or of the head and thorax were used; it was successful whether the insects, being infective, had fed recently on diseased or healthy plants. The method of obtaining the inoculum allowed of

no decision whether the virus had come from some internal organ (*e.g.*, the intestine) or from the blood of the insect.

I have attempted to obtain the blood alone by making a shallow puncture into the abdomen, thorax or leg, and exerting gentle pressure.* The results given in Table II show that inoculation of the blood of infective hoppers gives

Table II.—Inoculation of blood of infective active *C. mbila* into active *C. mbila*.

Experiment number.	Inoculum. Source.	Inoculum. History of infective insects.		Test insects. Number inoculated.	Test insects. Number infective.
			days		
1	Abdomen	On diseased maize	6	27	16
2	,,	,,	1	27	3
	,,	,,	2	21	1
	,,	,,	4	21	5
	,,	,,	21	27	12
3	,, *	,,	5	26	Nil
4	,, *	,,	14	21	7
5	Thorax	,,	23	21	16
6	Tibia of hind leg	,,	5	21	3
7	Abdomen	On healthy maize	15	25	8
8	,,	On healthy cane	10	27	10
9	,,	,,	29	26	4
10	Thorax	On healthy maize	8	23	6
11	,,	,,	30	19	Nil

* Nymphs.

positive results, whatever may be the site of the puncture or the recent history of the hoppers. In one experiment (No. 6) the blood was obtained by puncture of the tibia of a hind leg; the positive result obtained dispels all possibility that the virus in the inoculum used had come from some internal organ of abdomen or thorax accidentally punctured in obtaining the blood. The blood, unlike the contents of the rectum, still contains virus after the insect has fed on a healthy plant, although there is an indication of a falling off of virus concentration if the period on a healthy plant be prolonged (Table II, experiments 9 and 11). On the other hand, the virus concentration is at least fully maintained if the insects continue to feed on diseased maize (Table II, experiment 2).

In the foregoing experiments it was impossible to obtain the inoculum

* A colourless fluid is obtained from such a puncture. The assumption that this fluid is the blood seems to be justified. Occasionally it bears globules of fat, derived probably from the "fat body."

aseptically. It might be argued that the insect was naturally contaminated with virus externally and that it was from that source that the virus in my inoculum came. I have carried out a control experiment, in which blood was transferred from a non-infective insect to the surface of the abdomen of an infective insect, and then inoculated back again into the original insect. Negative results were obtained with 14 such transfers. Further control is provided by the entirely negative experiments, recorded later, in the inoculation of the blood of inactive hoppers fed on diseased maize. If an insect feeding upon a diseased plant were liable to be externally contaminated with virus, inactive insects would be expected to be as subject to contamination as active ones.

The time relations of the appearance of virus in the blood and the development of the power to cause infections were studied in the following experiment. *C. mbila* undergoes a "latent" or non-infective period following its first feed on a source of virus (Storey, 1928), similar to that demonstrated for a number of vectors of virus diseases (Bald and Samuel, 1931; Carsner and Stahl, 1924; Kunkel, 1926; Linford, 1932; Severin, 1921; Smith, K. M., 1931). Groups of 10 active hoppers were fed for 3 hours on diseased maize and then tested, still in groups, for successive 3-hour periods on healthy maize. This procedure gives a value for the "minimum latent period"* at the temperature of the experiment (a mean of 23° C.). It will be seen from Table III, experiment 1,

Table III.—Comparison of minimum latent period in groups of 10 active *C. mbila*, fed on streak-diseased maize, with the appearance of the virus in the blood of similar groups.

Test Periods. (Times from first exposure to virus.)

Hours :→	0–3	3–6	6–9	9–12	12–15	15–18	18–21	21–24
Experiment 1.—*Tests for latent period*—								
No. of groups tested	—	4	4	4	4	4	4	4
No. of groups infective	—	Nil	Nil	Nil	2	2	3	1
Experiment 2.—*Tests for virus in blood*—								
No. of insects inoculated	20	20	19	20	—	—	—	15
No. infective	Nil	Nil	5	7	—	—	—	5

* The exact interpretation of this value is uncertain. In different individual insects the duration of the latent period varies widely (Storey, 1928). The latent period of a group, as here determined, may be regarded as the period required for the most "rapid" member of the group to reach the stage of inoculating a minimum infective dose of virus within the time allowed for each test, or as the period required for the doses inoculated by ll members of the group to aggregate to a minimum infective dose.

that 2 out of 4 groups caused infections after 12–15 hours from the start of the experiment. The blood of 10 similarly treated insects was inoculated after each time interval into about 20 non-infective active hoppers. The results (Table III, experiment 2) demonstrate that the virus appeared in the blood in between 6 and 9 hours from the start of the first feed on diseased maize. It is thus seen that the appearance of virus in the blood of the insects precedes the development of the power to inoculate in feeding a minimum infective dose.

The blood of an active leafhopper that has been inoculated with the virus may contain the virus some time after the original inoculation. In one experiment, the blood of six active hoppers, that had been inoculated 14 days previously with the blood of infective hoppers and had successfully infected plants during the preceding 4 days, was inoculated into 18 non-infective hoppers. Of these one became infective. In a similar experiment, in which the inoculum was obtained from hoppers that had become infective as a result of inoculation, 19 days previously, with juice of diseased maize, 1 out of 23 hoppers became infective. There are thus indications that the virus may persist in the blood after inoculation, although the proportion of successes was less than with blood obtained from hoppers that had taken up the virus in feeding.

The Virus in Inactive Races of Vector Species.

Although uninjured inactive *C. mbila* will never transmit the streak virus in the course of feeding, yet they may be inoculated with the virus and may then become infective. By the inoculation of the juice of diseased maize into a normal puncture, 41 out of 105 inactive hoppers were made infective. The mean percentage of successes in the eight separate experiments from which these totals were obtained was $38 \pm 7 \cdot 0$. This percentage is significantly less than that obtained by similar inoculation of active hoppers. Heavy inoculation into the femur of a hind leg resulted in 5 infective hoppers out of 25. Thus inactive hoppers may be made infective by the introduction of the virus into the blood alone; but here again there is an indication that the inoculation is less effective than with active races.

Inoculated inactive insects, like active ones, produce infections irregularly and tend to become non-infective in course of time.

The normal behaviour of the virus in inactive leafhoppers has been studied by transferring inocula obtained from them to active hoppers. The general body contents of inactive hoppers proved to be virus-bearing only when taken

from the abdomen and only if the hoppers had recently fed on diseased maize (Table IV). The blood of uninjured inactive hoppers, after feeding on diseased

Table IV.—Inoculation of general body contents of inactive *C. mbila*, fed on streak-diseased maize, into active *C. mbila*.

Experiment number.	Inoculum.			Test insects.	
	Site.	History of insects.	days	Number inoculated.	Number infective.
1	Abdomen	On diseased maize	>2	16	4
2	Thorax	,, ...	>2	16	Nil
3	Abdomen	On diseased maize, then on healthy maize	5	15	Nil
4	Thorax	,, ,,	5	14	Nil
5	Abdomen	On diseased maize, then on healthy sugar cane, Co 205	6	26	Nil

maize, never contained the virus (Table V). The fluid from the rectum was found to contain the virus, if the insects had recently fed on diseased maize,

Table V.—Inoculation of blood of inactive *C. mbila*, fed on streak-diseased maize, into active *C. mbila*.

Experiment number.	Inoculum.			Test insects.	
	Site.	History of hoppers.	days	Number inoculated.	Number infective.
1	Abdomen	On diseased maize	3	26	Nil
2	,, ...	,, ...	4	28	Nil
3	,, ...	,, ...	6	31	Nil
4	,, ...	,, ...	14	18	Nil
5	,, *........	,, ...	4	20	Nil
6	,, *........	,, ...	5	26	Nil

* Nymphs.

but not if a period of feeding on healthy plants had followed the feed on a diseased plant (Table VI).

These results show that the inactive insect is able to take up the virus in feeding and to pass it through the intestine. But, in contrast to its behaviour in the active insect, the virus does not pass out from the intestine into the blood. The results obtained by the inoculation of the general body contents

Table VI.—Inoculation of contents of rectum of inactive *C. mbila,* fed on streak-diseased maize, into active *C. mbila.*

Experiment number.	Inoculum.		Test insects.	
	History of insects.		Number inoculated.	Number infective.
		days		
1	On diseased maize	2	28	8
2	,,	3	19	1
3	On diseased maize, then on healthy Co 205	5	24	Nil

conform to this explanation. For within the thorax the intestine is a narrow tube, through which food-material is believed to pass rapidly; in the abdomen the intestine enlarges to form the crop (fig. 5), whence, in the experiments of Table IV, the virus was doubtless obtained.

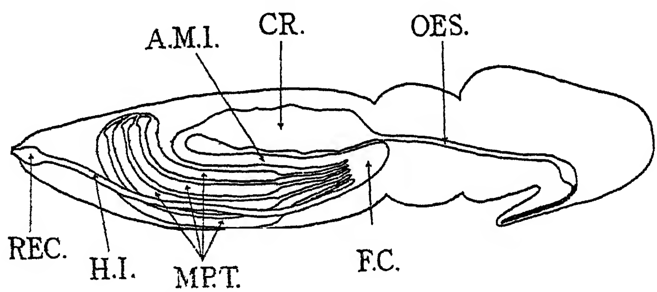

FIG. 5.—Diagrammatic vertical longitudinal section of *Cicadulina mbila,* showing digestive system. OES., œsophagus; CR., crop; A.M.I., ascending portion of mid-intestine; F.C., filter chamber; H.I., hind intestine; REC., rectum; MP.T., four Malpighian tubules. (These tubules undergo considerable convolutions, which are not depicted in this diagram.)

The rectal contents, which I have shown to contain the virus, may be used as an inoculum for the inoculation of inactive hoppers. In the experiment of Table VII the rectal contents of inactive hoppers, immediately after a feed on a diseased plant, were inoculated by a shallow abdominal puncture into the identical hoppers from which they were obtained. Two out of 21 hoppers were made infective in this way. A control series of hoppers was first gently pressed to cause an exudation at the anus and then a shallow abdominal puncture was made with a sterile needle. None of the control insects became infective. This experiment involved the *external* transfer of material from the rectum to the blood-fluid. The transfer may equally be done *internally*, by puncturing

Table VII.—Inoculation of inactive *C. mbila* with their own rectal contents, after feeding on streak-diseased maize.

Experiment number.	Inoculation method.	History of hoppers.	Tests.	
			Number inoculated.	Number infective.
		days		
1	Shallow puncture	On diseased maize . 6	21	2
Control	Shallow puncture (no inoculum)	,, ... 6	23	Nil

the rectum. Of 22 hoppers, recently fed on diseased maize and punctured by introducing a needle into the anus, 4 became infective (Table VIII, experiment 1). The hoppers may also become infective if the period of feeding on diseased maize is deferred until after the rectum has been punctured. In two experiments (Table VIII, experiments 2 and 3) this treatment was successful with 9 hoppers out of 35.

Table VIII.—Inoculation of inactive *C. mbila*, fed on streak-diseased maize, by puncture of rectum through anus with sterile needle.

Experiment number.	History of hoppers.	Tests.	
		Number inoculated.	Number infective.
	days		
1	On diseased maize before puncture 2	22	4
2	On diseased maize after puncture........... 2	14	5
3	,, ,, ... 2	21	4

These experiments prepare the way for the understanding of an observation made early in the course of this work. If inactive leafhoppers be punctured in the abdomen with a sterile needle, and particularly if the puncture be deep, some of these inactive leafhoppers may now behave as if they were active. That is, if the puncture be made immediately before or immediately after the hopper has fed on a diseased plant, that hopper may become infective. This experiment has been repeated many times and has indeed become a routine laboratory procedure for obtaining infective inactive hoppers. I summarize in Table IX a number of experiments on these lines. The proportion of

successful infections by this method is not high, but is sufficient to demonstrate the possibility of the process. An analysis of the figures of the several experiments showed no significant difference in the proportion of successes obtained by feeding the insects on diseased maize immediately before or after the puncture had been made. I would repeat that the inactive leafhoppers, if uninjured,

Table IX.—Inoculation of inactive *C. mbila* by abdominal puncture with sterile needle and feeding on streak-diseased maize.

History of insects.	Number of experiments.	Test insects.	
		Total inoculated.	Total infective.
Fed on diseased maize before puncture	9	211	31
Fed on diseased maize after puncture	14	271	57
Controls—Fed on diseased maize, not punctured	13*	353	Nil

* In many instances one control sample served for two or more experiments performed on the same day.

will never become infective by simple feeding on a diseased plant. Simultaneously with the majority of the experiments listed in Table IX, and in those that follow, a random sample of 20 to 30 hoppers was removed from the supposed inactive culture, fed on diseased maize, and tested in a group on a maize seedling (see Controls of Table IX). In no instance did infection follow the feeding of any control sample. In some instances the actual leafhoppers, subsequently used in the experiments, were first subjected to a similar control test and proved to be non-infective.

As further controls to the foregoing experiments, two series of 45 hoppers in all were punctured but not fed on a diseased plant, while a third series of 24 hoppers was anæsthetized only and then fed on a diseased plant. None of the control hoppers became infective. On the other hand an abdominal puncture was without effect on the normal transmission by active hoppers. Twenty-eight active hoppers were punctured and then fed on diseased maize; all became infective.

If the puncture was made into inactive leafhoppers, that had fed on a diseased plant and then for a period on a healthy plant immediately before puncturing, the hoppers did not become infective (Table X). If on the other hand the first feed on a diseased plant was postponed until 2 to 8 days after the puncture has been made, some of the hoppers became infective (Table XI).

The mean percentage of success in these experiments was, on the figures available, not significantly different from that obtained by feeding the punctured hoppers on a diseased plant at once. I shall show later, however, that a puncture made 40 days previously is no longer effective in causing an inactive hopper to become infective following a feed on a diseased plant.

Table X.—Inoculation of inactive *C. mbila* by abdominal puncture with sterile needle after feeding first on diseased maize and then on healthy maize before puncture.

Experiment number.	History of insects before puncture.		Tests.	
			Number inoculated.	Number infective.
		days		
1	On diseased maize, then on healthy maize	7	30	Nil
2	,, ,, ...	9	19	Nil
3	,, ,, ...	14	21	Nil

Table XI.—Inoculation of inactive *C. mbila* by abdominal puncture with sterile needle, followed by feeding on healthy maize and then on diseased maize.

Experiment number.	History of insects, after puncture.		Tests.	
			Number inoculated.	Number infective.
		days		
1	On healthy maize	2	19	2
	On diseased maize	2		
2	On healthy maize	8	16	2
	On diseased maize	2		
3	On healthy maize	7	20	3
	On diseased maize	2		

The position in which the puncture is made is important. A deep puncture into the anterior part of the abdomen is more effective than one near the tail (Table XII, experiment 1). A puncture made by a needle passed horizontally through the abdomen is probably less effective than a vertical puncture but more effective if it be near the dorsal surface than if it be near the ventral surface (experiment 2). A shallow puncture into the abdomen was successful

Table XII.—Inoculation of inactive *C. mbila* by puncturing with sterile needle in different places and feeding on diseased maize.

Experiment No.	Puncture.		Fed on diseased maize.	Test insects.	
	Nature.	Site.		Number inoculated.	Number infective.
1	Deep	Abdomen, anterior end	After puncture	23	8
	,,	Abdomen, posterior end	,,	27	Nil
2	Transverse	Abdomen, near dorsal surface	,,	27	2
	,,	Abdomen, near ventral surface	,,	24	Nil
3	Shallow	Abdomen, middle	Before puncture	25	1
4	,,	,,	,,	23	Nil
5	,,	Thorax	After puncture	9	Nil
6	No puncture	Abdomen compressed	After treatment	13	1

with 1 hopper only out of 48 (experiments 3 and 4). A puncture into the thorax proved fatal within 2 days to 38 out of 47 hoppers, but none of the surviving 9 hoppers infected a test plant (experiment 5). A severe compression of the abdomen, by a needle held transversely to the abdomen, without puncturing the exoskeleton, caused 1 out of 13 hoppers to become infective (experiment 6). This evidence of position is to be considered in relation to the anatomy of *C. mbila*. In fig. 5 is shown diagrammatically the digestive system of this species, which appears, in the features essential to the present argument, to be typical for this group of insects (*cp.* the description of *Cicadula sexnotata* by Dobroscky (1931)). The anterior dorsal portion of the abdomen, in which as I have shown the puncture is most effective, is occupied by the organ usually called the "crop," relatively large and consequently, it may be supposed, the most vulnerable portion of the intestine. It is suggested that penetration of the intestine by the needle is the necessary condition for an inactive hopper in these experiments to become infective.

Certain direct experimental evidence appears to support this suggestion. A series of 20 inactive hoppers, fed on diseased maize, was punctured deeply in the anterior part of the abdomen. Upon withdrawal from each inactive hopper the needle was immediately pricked into the abdomens of two non-infective active hoppers; that is, the fluid picked up by the needle in each inactive hopper was transferred to two active hoppers. Both series were then put on test. Of the first series, three hoppers became infective. Of the second series one pair of hoppers became infective, and these hoppers had been inoculated from one of the three inactive hoppers that became infective.

I have shown that the blood of an uninjured inactive hopper, fed on diseased maize, does not contain the virus. This was confirmed in a control experiment parallel to that described above, in which the blood of 20 inactive hoppers (selected at random from the sample of hoppers used in that experiment) was inoculated into active hoppers, none of which became infective. In the one successful experiment, therefore, the needle, in puncturing the inactive hopper, picked up the virus from some source other than the blood. I suggest that this source was the intestine, which is known to have contained the virus. Since in any case the amount of virus picked up is likely to be small, my failure to transfer virus from the remaining two inactive hoppers, that became infective, is not unexpected.

The evidence thus causes me to picture the infective inactive leafhopper of these experiments as one in which a part of the virus-bearing food ingested by mouth has passed out through a hole in the intestinal wall into the blood of the body cavity. That this is a true picture is supported by the abnormally distended appearance of many of the treated insects. I have been able, furthermore, to show experimentally that soon after puncture the blood may contain the virus. A series of 20 inactive hoppers was punctured and then fed for one day on diseased maize. Thereupon a little blood from each hopper was inoculated into two non-infective active hoppers. Tests of all these hoppers showed that two of the inactives of the first series (designated Nos. 4, 7), and four of the pairs of actives of the second series (Nos. 4, 5, 7 and 18), became infective. Thus two inactive hoppers, that became infective as a result of treatment, were shown to have had virus in their blood. Two others (Nos. 5 and 18), that failed to infect their test plants, also had virus in their blood. It may be noted, however, that these two inactive hoppers died within the first two days of the test, and may not have lived long enough to infect plants even though potentially infective.

Inoculated inactive hoppers may continue to show virus in their blood, so long as they remain infective. Two inactive *C. zeæ* (which behave in a similar manner to *C. mbila*—see below) were inoculated with the streak virus and proved to be infective in two successive tests. At the conclusion of the second test, 17 days after the original inoculation, the blood of each hopper was inoculated into five active *C. mbila*. One hopper in each series of five became infective. On the other hand, as I have already mentioned, the inactive hopper, made infective by one of the methods described above, will usually remain infective for only a short time. Hoppers that have ceased to be infective appear to contain no virus in their blood. An inactive hopper, pricked before feeding

on diseased maize, infected one test plant, but later failed to infect a second. Ten active hoppers, inoculated with its blood, were non-infective. In a similar experiment four inactive hoppers, made infective by puncture before feeding on diseased maize, were given a second test of 2 days' duration. In this second test two only successfully infected their plants, but the incubation of the disease in the plants was unusually prolonged. This feature I have come to associate with infections produced by hoppers that are on the point of becoming non-infective. The blood from the 4 hoppers inoculated into 30 active hoppers failed to cause any to become infective.

Inactive races of *Cicadulina zeæ* behave in a similar manner to *C. mbila*. Infective individuals of inactive *C. zeæ* were obtained by inoculation of the virus, and by deep puncture before and after the insects had fed on diseased maize (Table XIII). Again control tests confirmed the inability to transmit of uninjured random samples of the races of hoppers used in these experiments.

Table XIII.—Inoculation of inactive *Cicadulina zeæ* by various methods.

Experiment number.	Nature of puncture.	Source of inoculum.	Test insects.	
			Number inoculated.	Number infective.
1	Normal	Juice of diseased maize	25	12
2	,, ...	,, ...	28	3
3	Deep, abdominal	Fed on diseased maize *after*	33	5
4	,, ...	,, *before*	20	2

The Reinoculation of Insects that had become Non-infective.

I have already noted that an active insect, made infective by feeding upon a source of the virus, will normally remain infective up to the time of its death. Exceptionally, however, an insect will lose the power to cause infections. One such insect, after feeding on diseased maize, was infective for about three weeks and then failed in two successive weekly tests. After a second feed on a diseased plant, it infected plants in four weekly tests, but then failed in two tests. Thus after becoming non-infective this insect was not resistant to reinoculation by mouth. It will be noticed, however, that the original loss of infective power was not merely accidental, for even after reinoculation the same tendency to loss was manifested.

The inoculated insect, as I have mentioned, usually becomes non-infective

in course of time. The results shown in Table XIV prove that both active and inactive hoppers, successfully inoculated in various ways and later proved in two or more successive weekly tests to have become non-infective, may be successfully reinoculated by needle. Experiment No. 4 indicates, however, that a deep puncture, performed 40 days previously, is no longer effective in causing an inactive hopper to become infective by simple feeding on diseased maize.

Table XIV.—Reinoculation of *C. mbila*, originally made infective by inoculation and later proved to have become non-infective in two or more tests.

Experiment No.	Race.	Original inoculation.	Reinoculation.	Tests after reinoculation.	
				Number inoculated.	Number infective.
1	Active	Juice of diseased maize	Juice of diseased maize	5	3
2	Inactive....	,,	,,	4	3
3	,, ...	Punctured. Fed on diseased maize	,,	1	1
4	,, ...	,, ,,	Fed on diseased maize only*	1	Nil

* 40 days after the original puncture.

The Behaviour of Non-Vector Species.

The success obtained in the inoculation of inactive races of vector species led to experiments in the inoculation of other species, known to be unable normally to transmit the virus employed. For this purpose I inoculated species that are recognized to be vectors of viruses other than that used in the inoculation. There is every probability that a species able to transmit one virus is structurally not unfitted to transmit another virus, and it was therefore possible that its inability to transmit the second virus might be overcome by the treatment successfully applied to inactive races of vector species.

The results of all experiments to this end have been uniformly negative. *Cicadulina mbila* has been inoculated with the viruses of mosaic and stripe of maize. *Peregrinus maidis* and *Aphis maidis* have been inoculated with the virus of streak. The methods employed have been the direct inoculation of the virus by needle, and a deep puncture following feeding upon diseased plants. The evidence provided by the inoculation of *Peregrinus maidis* with

the streak virus is the most conclusive. The inoculation in different ways of 75 individuals of this species resulted in no infections of the test plants. Nevertheless, by inoculating into active *C. mbila* the rectal contents of *P. maidis*, recently fed on streak-diseased maize, I was able to prove that the virus might be ingested by this species and passed through the intestine. The blood, however, of *P. maidis*, fed on streaked maize, failed to make *C. mbila* infective. On the other hand, the inoculation of the rectal contents of *Aphis maidis*, fed on streaked maize, was unsuccessful.

Although the negative evidence provided by this series of experiments is not conclusive, there is a strong probability that the inoculation of non-vector species is impossible.

Discussion.

I have thus shown that after *Cicadulina mbila* has been in contact with a streak-diseased maize plant, the virus is present in the contents of the rectum. A part therefore of the virus, which must be assumed to have been ingested by mouth, passes through the length of the intestine. When, however, the contents of the rectum are naturally voided, the virus, for some reason that I am unable to explain, rapidly disappears.

In the active insect a part of the virus passes out from the intestine into the blood, where I have been able to demonstrate its presence. In the inactive insect, on the other hand, the virus is confined to the intestine and fails to reach the blood. If, however, the virus be introduced by inoculation into the blood of an insect of either class, that insect may become infective. Thus I am led to regard the presence of the virus in the blood of an insect as an essential step towards its becoming infective. It will be seen that this step precedes in time the development of the power to cause infections.

Since the introduction of the virus into an active insect's rectum is ineffective, it appears that the site of passage of the virus through the intestinal wall is located elsewhere than in the rectum and possibly the remainder of the hind intestine.

The inactive insect differs from the active one primarily in a property of the wall of the intestine that resists the passage of the virus. I have shown that this resistance may be overcome by a puncture of the intestine. This puncture may be made by needle through the rectum or crop or probably occasionally by simple rupture of the intestine following pressure on the abdomen. The evidence that I offer leaves little doubt that I have thus correctly interpreted the nature of the operation performed on these insects. The survival of an insect in which a hole in the intestine allows the food materials to flow out

into the blood implies a surprising hardihood. Nevertheless, I believe that to be a true statement of the condition of such insects, and that they will survive, sometimes to the normal span of life, is certain.

While the primary mechanism of the resistance of inactive *C. mbila* thus appears to lie in some property of the intestinal wall, some imperfect secondary mechanism must operate also. It is necessary to postulate this in order to explain the significant difference in the proportion of successes obtained in the inoculation of active and inactive insects. I can, however, offer no suggestion as to the nature of this secondary resistance.

Insects that have lost the power to cause infections are not resistant to reinoculation. If therefore the original loss of the virus were due to a resistance developed by the insect, that resistance could be only very weak. On the whole the available evidence rather suggests that the loss is due to an exhaustion of the supply of virus in the insect.

In these investigations I have thus traced the behaviour of the virus up to its appearance in the blood of the vector insect. It is generally believed that the saliva is the vehicle by which the virus is transferred from the insect into the plant. If this assumption be correct, it may be supposed that the blood is the vehicle by which the virus is transported from the intestine to the salivary glands. Investigations of the virus in the insect's saliva will be described in a later communication.

My studies of non-vector species, though of limited extent, suggest that they cannot be caused to transmit by treatments successfully applied to inactive races of *C. mbila.* Although *Peregrinus maidis* may take up the streak virus and may pass it through its intestine, yet, so far as my experiments show, no treatment of the insect will cause it to transmit this virus. Nor was I more successful in attempts to cause *C. mbila* to transmit the stripe and mosaic viruses. The inability of non-vector species to transmit a virus appears to rest upon a more complete mechanism of resistance than that of inactive races of *C. mbila.*

This work has been carried out at the East African Agricultural Research Station, Amani, East Africa. I acknowledge the help that I have received from my assistant, Mr. R. F. W. Nichols.

Summary.

(1) This paper deals primarily with experiments in the mechanical inoculation of insects with plant viruses. The method employed was the introduction

of the virus into a puncture of the abdomen or leg made with a finely pointed needle or glass micro-pipette. A large proportion of the insects survived this treatment.

(2) The inoculation of active races of *Cicadulina mbila*, the vector of streak disease of maize, was successful, when the inoculum introduced was the juice of diseased maize seedlings, fresh or kept for four but less than eight days, undiluted or diluted with distilled water by 10^{-2} (rarely, by 10^{-3}).

(3) Inoculation by causing the virus fluid to be drawn up into the rectum *per anum* failed.

(4) By the inoculation of the appropriate fluids, it was shown that the virus was present in infective active *C. mbila*, (*a*) in the contents of the rectum, if the insect has recently fed on a diseased plant, but not otherwise, (*b*) in the general contents of thorax or abdomen, and (*c*) in the blood, whether the insect had fed recently upon diseased or healthy plants. The virus was not found in the naturally voided fæces. The appearance of the virus in the blood preceded in time the development of the power to cause infections.

(5) Inactive races of *C. mbila*—normally unable to transmit the virus—were made infective by needle inoculation with the streak virus. The proportion of successes was, however, significantly less than with active races.

(6) After a feed on a diseased plant, the inactive insect was found to have the virus in its rectum, but never in its blood.

(7) A simple puncture of the abdomen with a sterile needle, either following or followed by a feed on a diseased plant, sometimes caused inactive *C. mbila* to become infective. By comparison of the efficacy of different positions for the puncture, it was concluded that the treatment was successful only if the needle had penetrated some part of the intestine.

(8) Inactive races of *Cicadulina zeæ* proved to be susceptible to inoculation with the streak virus by the methods successful with *C. mbila*.

(9) *C. mbila* was not successfully inoculated with the viruses of maize stripe and mosaïc diseases; nor *Peregrinus maidis* and *Aphis maidis* with the virus of streak.

(10) It is concluded from these observations that in active *C. mbila* the streak virus, entering the intestine by mouth, passes through the intestinal wall into the blood; and that, in the inactive insect, the cells of the intestinal wall resist the passage of the virus. It is recognized that there may be some secondary mechanism of resistance; nevertheless in many inactive individuals, once the barrier of the intestinal wall has been passed, the virus behaves as in an active insect.

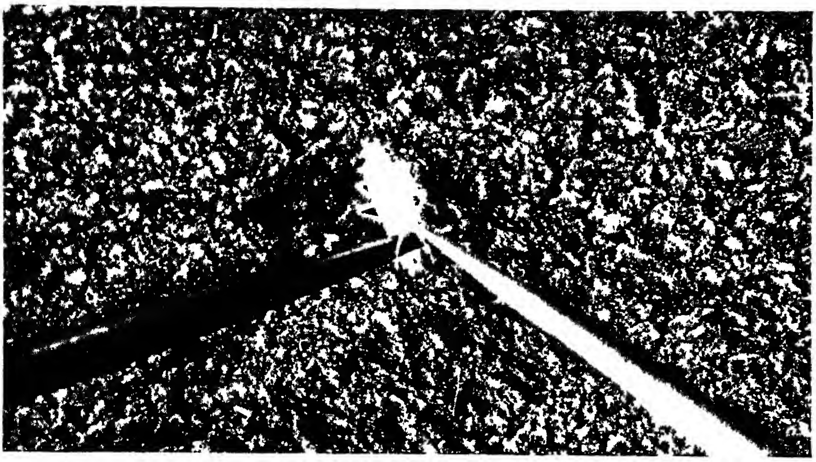

Fig. 1.

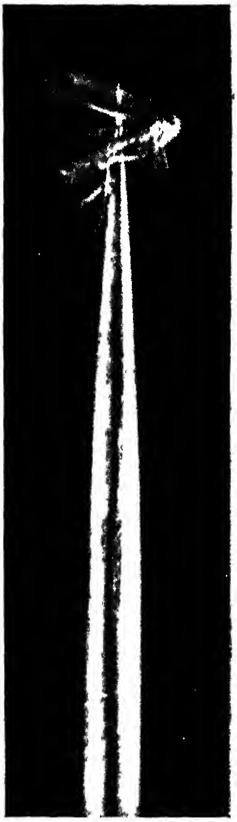

Fig. 2.

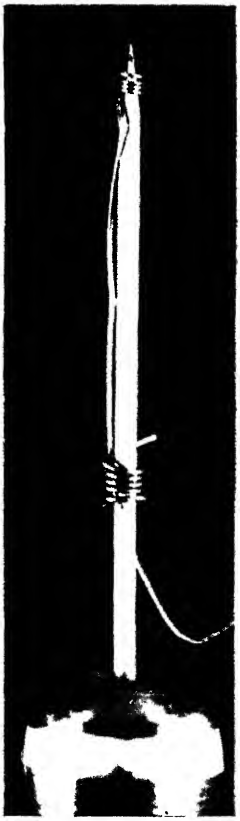

Fig. 3.

Fig. 4.

(*Facing p.* 484.)

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DESCRIPTION OF PLATE.

FIG. 1.—The method of inoculation of *Cicadulina mbila*. The insect is steadied by means of the "holding needle" on the left, and punctured in the abdomen with the "inoculating needle" on the right. × 7.

FIG. 2.—The method of "deep inoculation" of *Cicadulina mbila*. The point of the needle is thrust right through the anterior part of the insect's abdomen. × 7.

FIG. 3.—The "reservoir needle" used for "heavy inoculations." × 3.

FIG. 4.—The method of inoculation of *Cicadulina mbila* by means of a micro-pipette. × 7.

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Factors affecting the Breeding of the Field Mouse (Microtus agrestis). *Part* III.—*Locality*.

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(Communicated by E. S. Goodrich, F.R.S. Received May 25th, 1933.)

General Results of Observational Work.

The first two papers in this series (1932, *a* and *b*) were descriptions of experimental work. This third paper is based upon observation.

In order to study the effect of latitude upon the starting and stopping of breeding, we chose three areas in a nearly north and south line. The most northerly was about 360 miles from the most southerly, and the other area was roughly half way between them. All three areas were elevated grassland belonging to the Forestry Commission, whom we must thank for their kind co-operation, without which the investigation would have been impossible. The most northerly area was at Huntly in Aberdeenshire. The intermediate area was at Newcastleton in Roxburghshire. The most southerly area was at Corris in Merionethshire (see fig. 1). The Aberdeen and Roxburgh areas were at elevations of about 700 feet, and the Merioneth area at between 500 and 600 feet.

Microtus agrestis was caught in each area as nearly as possible every month from January, 1930, to December, 1931. Deep snow and other causes occasionally prevented the monthly trapping in one or other area. On the average 32 *Microtus* were caught each month during these two years in Aberdeen, 29 in Roxburgh, and 43 in Merioneth. The total number of mice caught in all three areas was precisely 2500. The mice were caught in breakback traps with wooden tongues, placed unbaited across the runs. *Microtus*, unlike other mice, is unattracted by any bait. The trapping in Aberdeen was supervised by Mr. J. Clarke, in Roxburgh by Mr. J. Macintyre, and in Merioneth by Mr. J. Lomas. We cannot exaggerate our indebtedness to these helpers, who kept up a constant monthly supply of mice in all weathers except when the traps could not be set owing to deep snow. The dead mice were sent by post to Oxford, where each was subjected to a routine examination.

Each mouse was first weighed in grams. Weights were recorded like ages ; thus any mouse weighing 20 gm. but less than 21 was recorded as 20 gm.

This method is as precise as the giving of the weight to the nearest gram, provided the method used is explained. It has the advantage that a half-gram weight need not be used. Weighing is thus quicker, which is an advantage in dealing with a large number of animals.

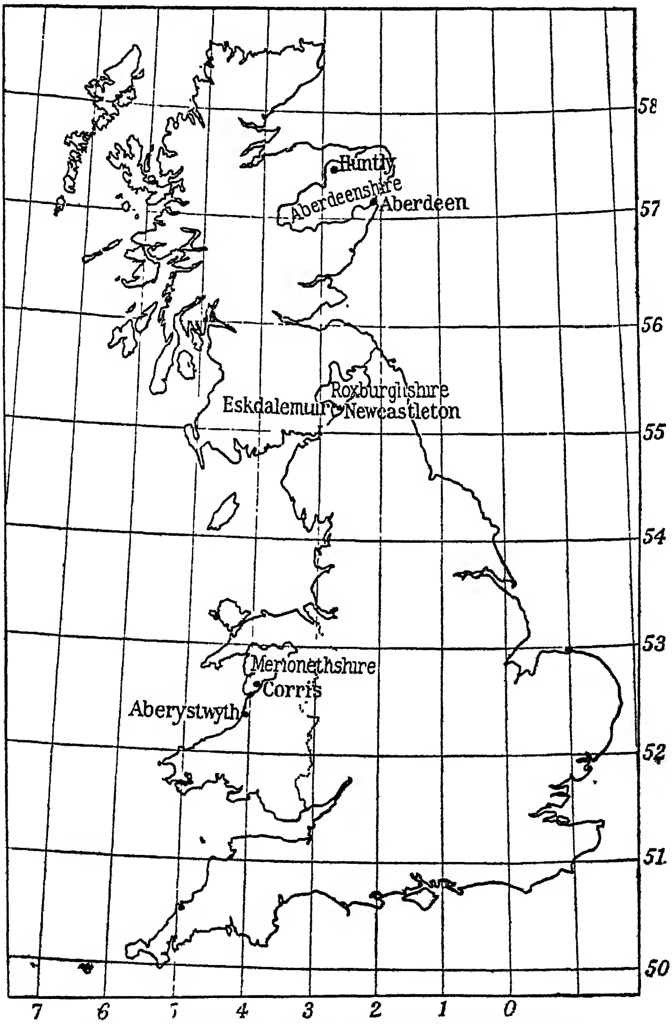

FIG. 1.—Collecting areas and meteorological stations.

In males the testes were weighed to 10 mg. (again on the "age" principle, to save time). The vesiculæ seminales were recorded as 100 mg. or more, or 10–99 mg., or less than 10 mg.

Males were regarded as fecund if the vesiculæ seminales weighed (both together) 100 mg. or more. This figure was selected as a result of previous

studies made by one of us on *Microtus* caught at Oxford during the breeding season. 80 males were caught with vesiculæ weighing 100 mg. or more; every one of these had sperms in the epididymis. 105 males were caught having vesiculæ weighing less than 100 mg., of these only 7 had sperms in the epididymis. Of these 105, 95 had vesiculæ weighing less than 20 mg., and no sperms at all in the epididymis. It is evident that the vesiculæ grow very rapidly between 20 and 100 mg., and that the possession of vesiculæ weighing 100 mg. or more is a good test of fecundity.

In females the vulva was recorded as perforate or imperforate. The perforation of the vulva was used as the criterion of fecundity. The number of embryos in pregnant females was recorded. The embryos were weighed, and the weight subtracted from the crude body-weight to give the actual body-weight of the female. In this paper the weights of females always mean weights without embryos.

Fig. 2 shows the body-weight and fecundity of all the males from all three areas in each month of the two years of the investigation. In this figure a horizontal line indicates a fecund mouse, and a vertical line a non-fecund mouse. Much the most striking point brought out by the figure is that the fully grown males of one year do not live over to the next year. By October most of these old males are dead. Some of them are caught in a non-fecund condition before they die off. It is conceivable that a few adult males sometimes manage to live over the winter, but it is certain that the great majority do not. During the winter the species is represented, so far as males are concerned, only by small, non-fecund individuals less than a year old. So far as we know, this is a phenomenon which has never been described in any mammal, though, of course, many insects over-winter in the larval condition.

About March, the young males which have over-wintered begin to become fecund and grow rapidly, as the figure shows. By April practically all are fecund, and maximum body-weights are attained in May or June.

The new season's young mice begin to be caught in our traps about May, but the figure appears to prove that most of the mice born in one year do not attain their full size or fecundity till the next year. Very few mice are caught weighing about 23 gm., though this is about the average weight, and one would have expected to find at least as many at this weight as at any other. (It is just possible that they may either grow extremely rapidly past this particular weight, or that they may become inactive and thus avoid capture at this stage of growth.)

Fig. 3 shows the growth of the testes of mice which were born in 1930 and

over-wintered in an immature condition. (In the graph the weight "0" indicates, of course, any weight less than 10 mg.) The mean weight of the testes of all fecund males caught in the months of May, June, July and August was 483 gm.

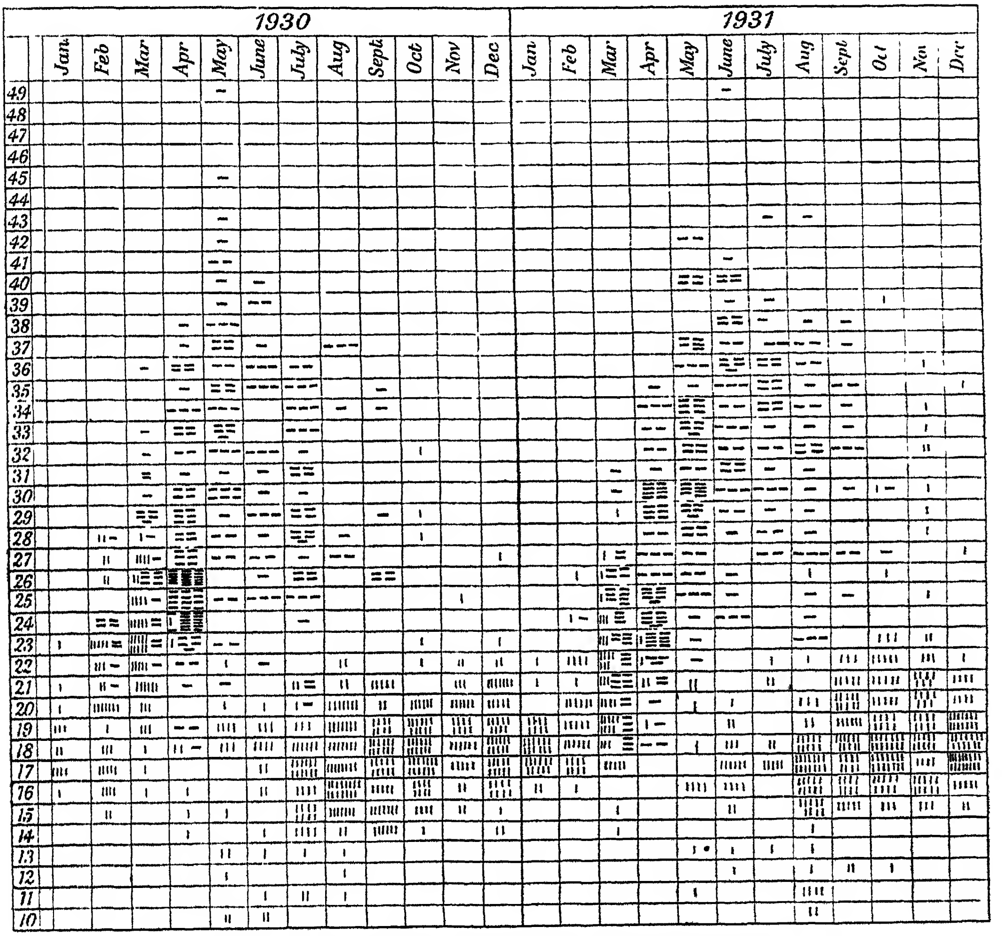

FIG. 2.—Diagram showing body weight and fecundity of males.

Fig. 4 shows the body weight, fecundity and pregnancy of all the females from all three areas. Each dot represents a pregnant female, each line a non-pregnant one. A horizontal line represents a fecund female (*i.e.*, a female with a perforate vulva); a vertical line represents a non-fecund female. The figure makes it clear that, as with the males, the big females die off before the winter; but there is this difference from the males, that a considerable number

of females that are large enough to be fecund survive. Pregnancies first occur in March or April, and are found for the last time in September or October.

A great deal of information may be obtained from a careful study of figs. 2 and 4.

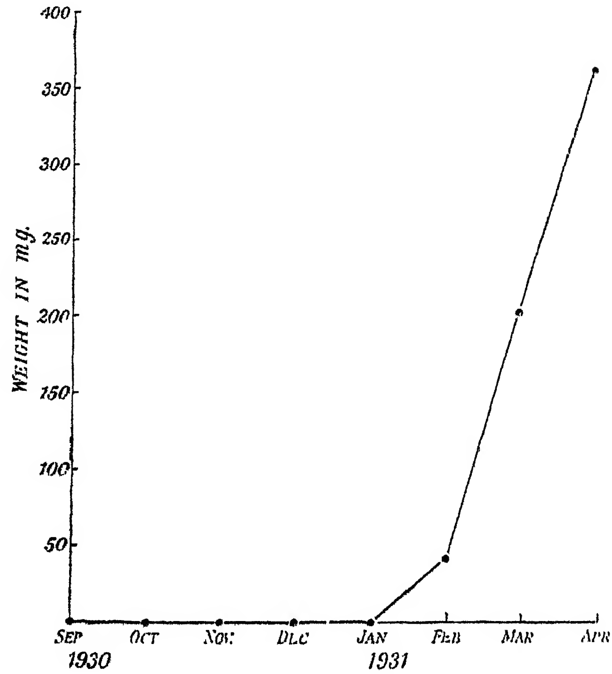

Fig. 3.—Growth of testes.

Differences in the Breeding Season in Different Localities.

The Start of the Breeding Season. Males.—In 1930 breeding definitely started latest in the most southerly of the three stations, Merioneth. 41 males were caught there in February and March, of which none was fecund. (From the beginning of April onwards practically all were fecund.)

Breeding condition was attained much earlier in Roxburgh. In February, 9 fecund males were caught, out of a total of 25. All the 18 males caught in March were fecund except 1.

In Aberdeen snow prevented the catching of mice in February. In March, 34 males were caught, of which 8 were fecund.

It appears that breeding condition was attained first in Roxburgh, then in Aberdeen, and last of all in Merioneth.

In 1931 breeding was later once again in Merioneth than in the more northerly stations. Of 29 males caught there in February, none was fecund, while 1 of the 5 caught in Roxburgh was fecund. (None was caught in Aberdeen owing to snow.)

In March there were males in breeding condition everywhere, but in Merioneth only 4 out of 26 caught were fecund, while in Roxburgh about half (13 out of 24) were fecund, and in Aberdeen 23 out of 29.

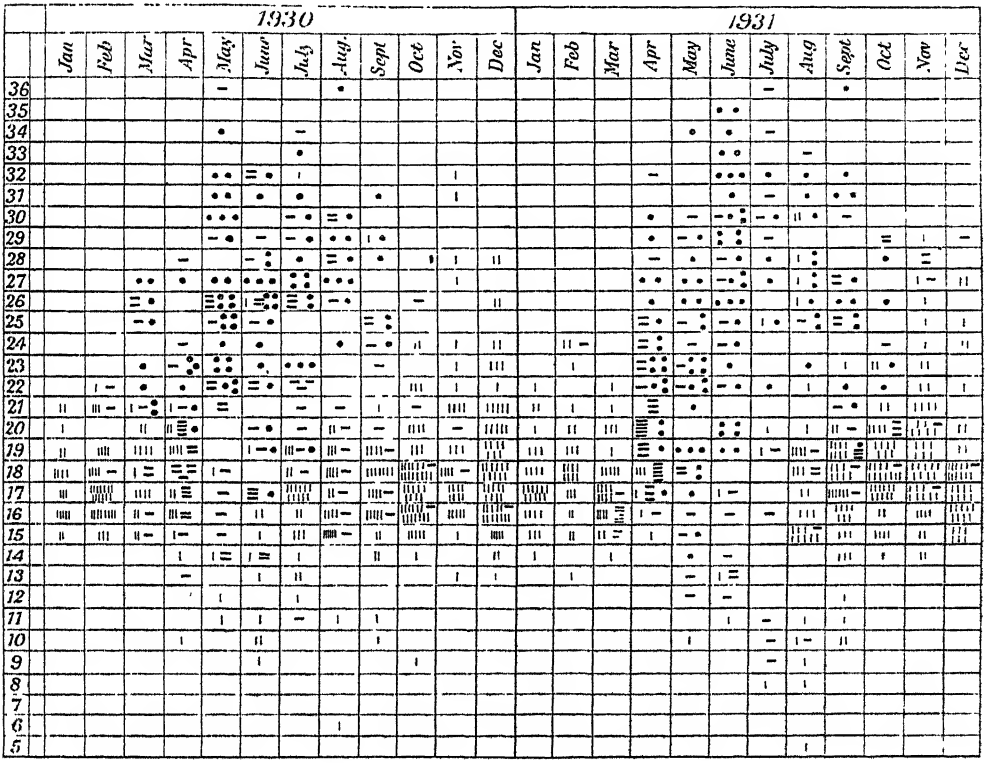

Fig. 4.—Body weight, fecundity and pregnancy of females.

The general conclusion is that in both years males attain breeding condition later in Merioneth than in the more northerly stations.

The Start of the Breeding Season. Females.—In 1930 pregnancies came earliest in Roxburgh. Eight out of the 12 females caught there in March were pregnant. In this month none of the 14 females caught in Merioneth or of the 6 caught in Aberdeen was pregnant. In April breeding just started in Merioneth and Aberdeen. In Merioneth there were 2 pregnancies among 12 females, and in Aberdeen 1 among 21. In May breeding was general in all localities.

In 1931 there were no significant differences between the three localities. Breeding was general in April, but no pregnancy was found before April with the exception of one in Aberdeen on March 26.

The End of the Breeding Season. Males.—In both years breeding condition was retained to a later date in the most northerly station, Aberdeen, than in the other stations. In 1930 there was still a little fecundity in all three stations in September, but none in October. In September a quarter of the males (5 out of 20) were fecund in Aberdeen, while in Roxburgh only 1 was fecund out of 17 and in Merioneth only 1 out of 25.

In 1931 there was still a little fecundity at all three stations in September, but in October there was no fecundity except at Aberdeen, where 2 out of 27 were fecund. None of the 18 males caught in Roxburgh nor the 28 in Merioneth was fecund.

The End of the Breeding Season. Females.—In 1930 there were pregnancies in all three stations in September, but none in October. In Roxburgh and Aberdeen, but not in Merioneth, fecund females were still found later than September.

In 1931 the last pregnancies in Merioneth were in September. There were still pregnancies in October in Roxburgh and Aberdeen, and there were a few fecund females so late as December in Aberdeen.

The main general conclusion that we draw is that in both sexes breeding condition is attained latest in the most southerly station, and is retained latest in the most northerly station.

Correlations between Climate and Reproduction.

The climate of the three localities is shown in fig. 5. There are no meteorological stations on the collecting areas, so we have obtained information from the nearest station to each, namely, Aberdeen, Eskdalemuir for Roxburgh, and Aberystwyth for Merioneth, fig. 1. The first and last stations are unfortunately at considerably lower altitudes than the trapping areas.

Orton has shown (1920) that in many marine invertebrates breeding starts when the temperature of the sea rises to a certain figure, and continues as long as it remains above that figure. Temperature is often regarded as the main factor controlling the breeding seasons of vertebrates also, although there is no direct evidence to show this. We have been able to prove definitely that *Microtus* does not simply breed above a certain temperature. In both sexes fecundity starts in the very coldest month of the year, or shortly afterwards (February or March). It stops when the temperature is still high. September

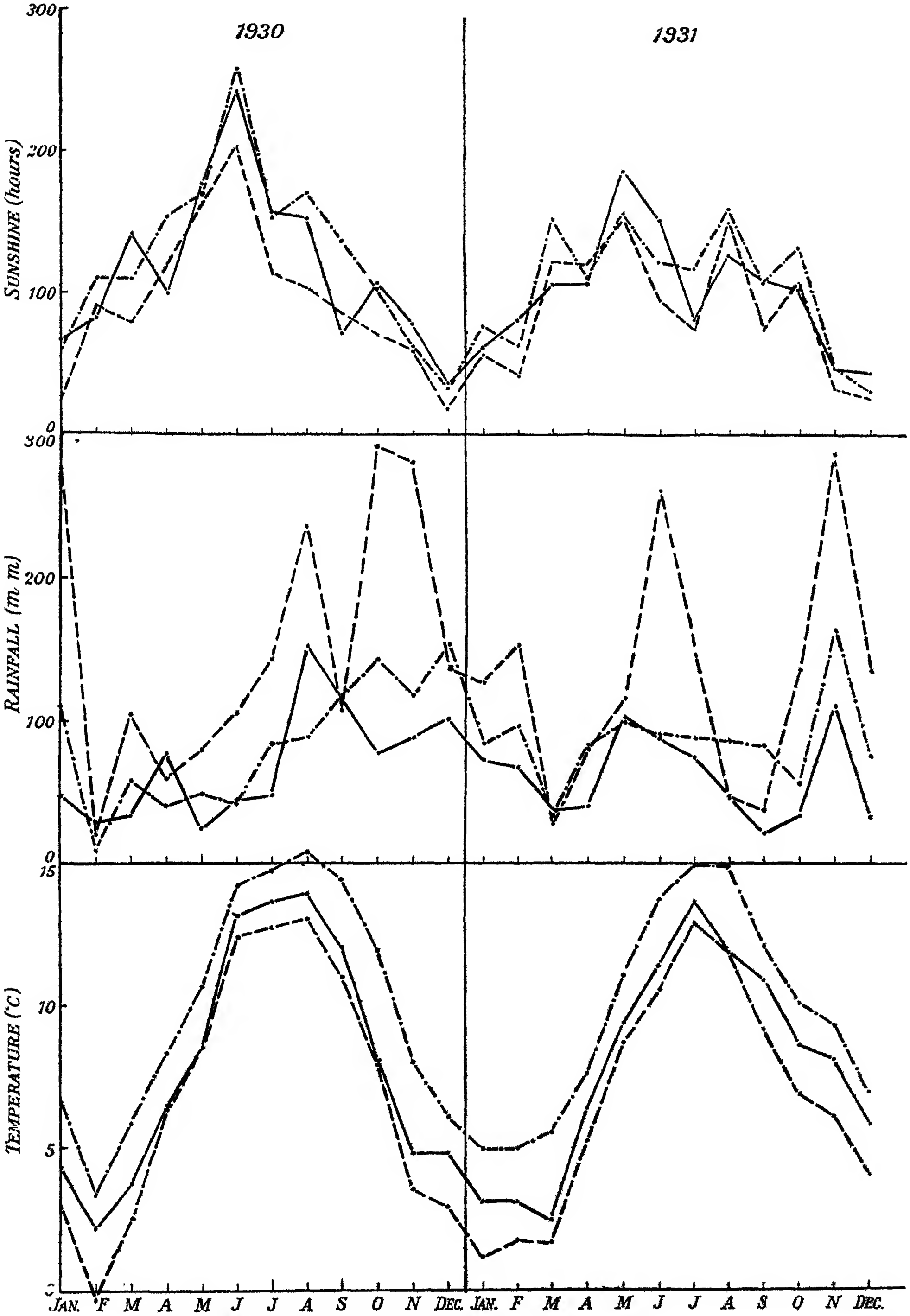

FIG. 5.—Meteorological records of stations nearest the trapping areas.

— · — · — Aberystwyth ; — — — Eskdalemuir ; ———— Aberdeen.

is the last breeding month, pregnancies being rare in October. Yet the temperature of October is roughly the same as that of May, in which month reproduction is at its height.

We have not been able to find any correlation between breeding and rainfall.

We have found a remarkable general correlation between the hours of sunshine and reproduction. The upper graph of fig. 5 shows the number of hours of sunshine in each locality. (We have not thought it worth while to adjust the figures to make up for differences in the lengths of the months.) In general mice were breeding in those months in which there were more than about 100 hours of sunshine, and were not breeding when there were less. There were some exceptions to this rule, but there is far closer correlation with hours of sunshine than with any other climatic factor investigated. In 1930 breeding started and ended earlier than in 1931. In 1930 pregnancies were found from March to September; in 1931 from April to October. If one holds a sheet of paper over the sunshine graph in such a way as to hide the part of it below 100 hours per month, it becomes immediately obvious that the season of long hours of sunshine came earlier and ended sooner in 1930 than in 1931.

The differences between the times of breeding in the different localities cannot be correlated with the hours of sunshine in each, but it must be remembered that the meteorological stations were not on the actual trapping areas. There is, of course, no proof that the hours of sunshine affect breeding; but the facts are suggestive, especially in view of our previous experimental work on the effect of light on reproduction in *Microtus* (1932, *a*).

Summary.

(1) The breeding of *Microtus agrestis* was studied in three areas about equally distant on a nearly north and south line about 360 miles long. Mice were collected each month for two years and the reproductive organs studied. Altogether 2500 mice were dissected.

(2) The breeding season lasts from February or March to September or October.

(3) During the winter the species is represented, so far as males are concerned, entirely by small, immature specimens less than a year old. These become fecund next spring and die in the autumn or early winter. The majority of the mature females also die in autumn or early winter.

(4) Breeding starts latest in the most southerly of the three areas, and continues latest in the most northerly. This has not been explained.

(5) There exists a general correlation between the hours of sunshine per month and the breeding condition of the mice. No correlation was found between temperature or rainfall and breeding.

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The Liminal Brightness Increment as a Function of Wave-Length for Different Conditions of the Foveal and Parafoveal Retina.

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1. *Introduction.*

In this paper will be given the results of measurements of the least perceptible difference of brightness between a test patch and its immediate surrounds, the difference of brightness being produced by monochromatic light of various wave-lengths throughout the spectrum. Observations have been made both for foveal and 5°-parafoveal vision of the test spot, and with the eye in various conditions of adaptation.

In the early studies of the brightness difference threshold (b.d.t.), for example in König and Brodhun's (1903) classical research, the subject viewed a photometric field of the same colour (spectral composition) throughout, but divided into two halves whose brightnesses were adjusted to the critical difference. Except for the rather small photometric field (6°×4·5° for König and Brodhun) the visual field was dark. The results were expressed by giving Fechner's fraction $\delta B/B$* which is nearly constant and independent of colour at high brightnesses and which is independent of the units in which the brightnesses are expressed. Later workers (Cobb, 1916; Schjelderup, 1920; Emerson and Martin, 1925; and others) studied the effect of surrounding the bipartite test field with an extended area of brightness B_s. Schjelderup expressed his results in the form, $\delta B = aB + bB_s$, where a and b are constants. Cobb, Dittmers (1920), Martin and Emerson found that $\delta B/B$ was minimal when $B = B_s$. A further group of investigations have dealt with the effect of a bright patch of light (or glare source) on the brightness difference threshold (Bordoni, 1924; Luckiesh and Holladay, 1925; Holladay, 1926; and Stiles, 1929). The tendency in the more recent work is to depart from the small bipartite photometric field which the subject adjusts to a threshold difference of brightness. Stiles, for example, used a square field of approximately 40° side at whose centre a 2° diameter test spot was formed. The criterion was

* For list of symbols, see p. 524.

not "is there a difference of brightness between the centre test spot and the rest of the field ?" but "is the test spot there or not ?"

For the more varied conditions of stimulation of the eye, including large fields, surround brightnesses or glare sources, the use of Fechner's fraction $\delta B/B$ to express the results of measurements of the b.d.t. is no longer appropriate and we revert to the b.d.t. itself. In fact, we are led to adopt a more general notion of the b.d.t. Suppose the stimulation over the whole visual field to be fixed both in colour (spectral composition) and intensity and let us call this the conditioning stimulation. The colour and intensity of the conditioning stimulation may vary from point to point in the field. For any given conditioning stimulation, suppose we apply over a small area about a particular point P in the field a uniform stimulation of any spectral composition symbolized by $\{e_\lambda\}$ but of variable intensity u. If U is the critical value of u at which the presence of the test patch can just be detected or just not be detected by the subject, then U corresponds to the b.d.t. at the point P for the given conditioning stimulation and for the particular test stimulus employed. This definition excludes the case when the test patch is darker than its immediate surrounds and it also excludes measurements, such as Troland's, of the brightness difference threshold between different spectral colours. To avoid ambiguity, we shall use the term "liminal brightness increment (l.b.i.)" for the threshold quantity U defined above.

The liminal brightness increment will depend on various factors which can be classed under three headings :—

(*a*) The position in the visual field of the point P.

(*b*) All the variables which determine the conditioning stimulation. If the eye has not become completely adapted to the stimulation prevailing at the time of observation, its previous light history must also be included in the conditioning stimulation.

(*c*) All the variables determining the test stimulus and the method of taking observations and deriving the critical value of u.

In the experiments here described, the test stimulus consisted of a monochromatic patch of wave-length λ, which was variable, the other factors under (*c*) being kept the same throughout. Thus for each conditioning stimulation studied, we have obtained the law of variation of U with λ. For the eye in equilibrium with zero conditioning stimulation, Abney and Watson (1916) obtained for a number of subjects the variation of U with λ both for foveal and parafoveal points. Our observations for this condition are in general agree-

ment with Abney and Watson's results for their larger class of subjects. It is at high brightness conditioning stimulations, however, that our results exhibit a novel and significant character. Expressing U in energy units and plotting 1/U (or log 1/U) against λ the resulting curve has been found to have maxima at approximately 0·44 μ and 0·54 μ and a third maximum or bulge in the neighbourhood of 0·60 μ. It is likely that these three maxima are in some way connected with the three mechanisms postulated by the trichromatic theory.

Accounts of experimental studies on somewhat similar lines to those of the present paper have recently been published by Roaf (1932). We have followed Abney and Watson in expressing our thresholds in energy units, whereas Roaf expresses his intensities as multiples of absolute threshold values, and as the magnitudes of the latter are not given any quantitative comparison with the present results is difficult. Roaf's interpretation of his results appears to us to be unduly influenced by his choice of intensity units.

2. *Observational Conditions.*

The subject employed monocular vision, the idle eye being kept in total darkness. The observing eye was applied to a circular artificial pupil of 2 mm. (later 3 mm.) diameter. Five types of conditioning stimulation were studied :—

Conditioning stimulation I.—Zero brightness throughout the field.

Conditioning stimulation II.—An approximately circular area of diameter (N) = about 30° (reduced to 6° for some measurements), of uniform brightness B_s except at the centre, where there was a circular centre field 1·28° in diameter, of zero brightness. Beyond the extended area of brightness B_s the brightness graded off rapidly to zero over the rest of the field. The test spot was formed within the dark centre field.

Conditioning stimulation III.—Zero brightness throughout the field except for a bright patch (or glare source) of 0·8° diameter placed 3° above the test spot, and producing an illumination E at the eye (brightness of glare source = B_G).

Conditioning stimulation IV.—Conditioning stimulations II and III superposed.

Conditioning stimulation V.—As for II, except that the centre field instead of being dark was occupied by a uniform brightness B.

Before observations were commenced, the subject viewed the given conditioning stimulation for a sufficient time for the eye to become adapted. This entailed a preliminary period of observation of 1 hour for the conditioning stimulation I.

The test stimulus consisted of a rectangular patch of approximately monochromatic light of 0·7° horizontal and 0·16° vertical side. For foveal observations, the test spot was exposed continuously and its brightness was made to increase steadily from a very low value until the subject signalled that he could just see it, and then, from a value at which it was easily visible, its brightness was made to decrease steadily until the subject signalled that he was just unable to see it. For parafoveal observation, the test spot was exposed for one second every four seconds, the intensity increasing (or decreasing) by a constant proportion in successive exposures. This method was adopted to avoid the spontaneous disappearance of objects seen parafoveally on prolonged fixation.

For foveal observation the subject directed his gaze at the centre of the 1·28° centre field where the test spot was formed. For parafoveal observation a weak point source of light adjusted to be just visible at the fovea, was placed 5° to the right of the test spot. The subject fixed his eye on this point source so that the test spot fell on a patch of retina 5° (external angle) out in the parafovea. One subject (W. S. S.) used his left eye, the other (B. H. C.) his right eye throughout the work.

A diagram of the visual field of the subject is shown in fig. 1.

3. *Apparatus and Technique of the Measurements.*

Fig. 2 gives the general plan of the apparatus. This consists of a wooden cube of 52 cm. side with a conical projection carrying the artificial pupil O at which the subject applies his observing eye. Opposite the observing aperture is the background screen S which was whitened by smoking with magnesium oxide. The screen S is illuminated by the images of the filaments of two 1500-watt projection lamps C, formed by the large condenser lenses N and the spherical mirrors E. The lamps were run at their rated voltage (100 volts) and after some blackening of the bulbs had occurred in use, a brightness of S of approximately 300 candles/sq. ft. was obtainable. Lower wattage lamps were used for lower brightness levels. In the space between the condenser lenses and the apertures in the cube, neutral filters I could be placed to modify the screen illumination as desired. Owing to the considerable generation of

heat when using high wattage lamps, it was found necessary to insert between the lamps and the condenser lenses, water cells W through which cold water was circulated continuously. The angular diameter of the area of approximately uniform brightness seen by the subject equalled about 30°.

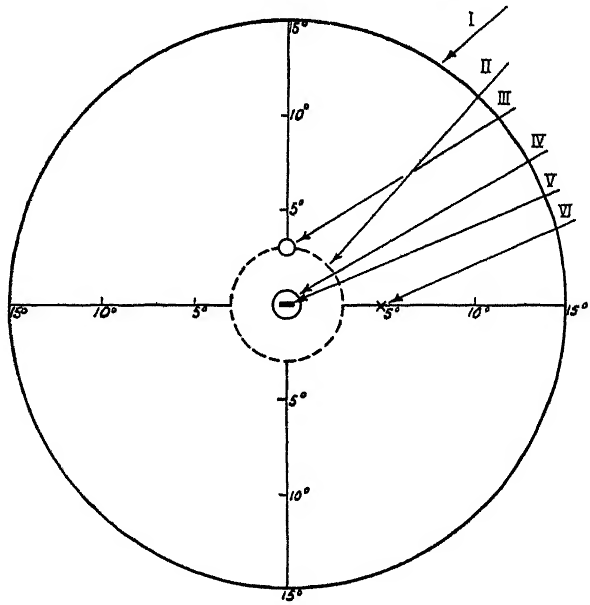


Fig. 1.—Visual field of subject.

I. Outer limit of large surround; II. Outer limit of small surround; III. Glare source; IV. Centre field; V. Test spot; VI. Point on which eye is fixed for parafoveal measurements.

At the centre of S a circular hole H of 1·25 cm. diameter subtending 1·28° at the eye, formed the centre field in which for conditioning stimulations I, II, III and IV the brightness was zero.

To study the effect of a brightness in the centre field (conditioning stimulation V) a plate of plain glass R_1 set at 45° to the rays passing through the spectrometer was arranged to reflect the light from an opal lamp A through the lens L_1 and the aperture of the centre field into the subject's eye. The subject then saw the centre field filled with a uniform brightness, on which the test spot was superposed. The surround was given a brightness of about the same value as that of the centre field so that the effect of a uniform background was reproduced.

The glare source for conditioning stimulation III was formed by the image of the plate of a 500-watt pointolite Q_1, exposed to the subject's view by a

small hole G in the background screen. The diameter of the glare source subtended an angle of about 0·8° at the subject's eye. In fig. 2 for convenience the glare source is shown at the side of the small centre field whereas in reality it was placed vertically above the latter. A water cell and, when

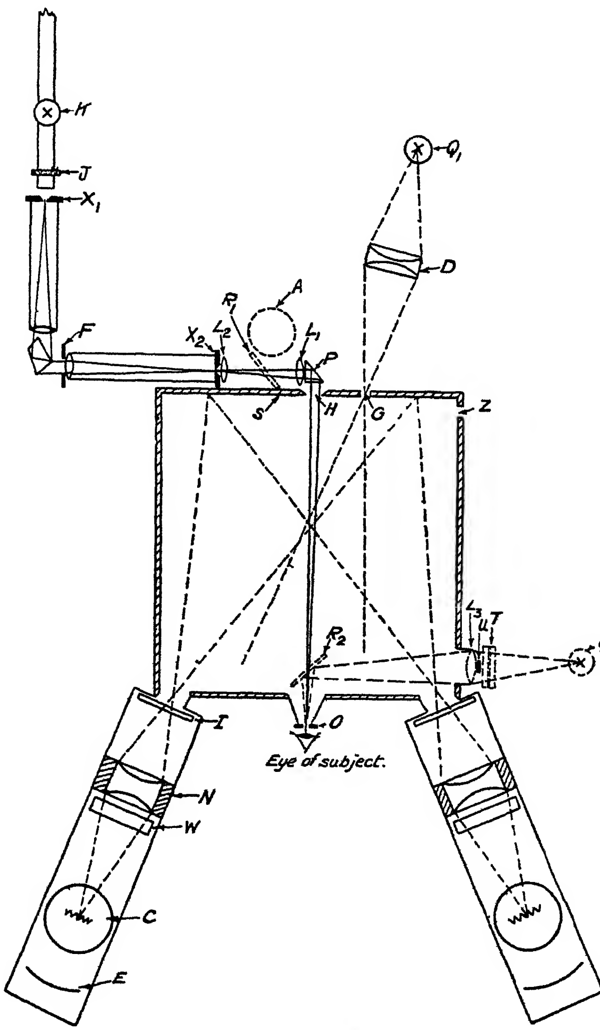


FIG. 2.—Diagram of apparatus.

necessary, neutral filters were placed between the glare source condenser D and the aperture G in the background screen.

In investigating the effect of a coloured conditioning stimulation, it was necessary to start with a very intense white stimulation so that when the colour

filter with its low transmission was interposed, the resulting colour stimulation was sufficiently intense to elicit characteristic effects. A different method of obtaining a bright surround was therefore adopted. The lens L_3 focussed an image of the plate of the pointolite Q_2 on to the artificial pupil at O, the light being reflected by a plate of plain glass R_2 set at an angle of 45°. The subject with his eye applied at O then saw by Maxwellian view a circular patch of high brightness of 6° diameter situated apparently in the plane of the background screen. A small circular opaque stop u, produced a 1·28° dark centre field at the centre of the 6° bright patch seen by the subject. The test spot was formed, as usual, in the centre of this 1·28° dark centre field.

By removing the opaque stop u, conditioning stimulation V with $B = B_s$ was obtained.

To specify completely the conditioning stimulation, it is necessary to give both the intensity and the relative energy distribution of the brightnesses involved. What we shall refer to as "white light" is the radiation emitted by the 1500-watt projection lamps or by the pointolites, and for this purpose we shall omit from consideration the slightly selective transmissions of the water cells and condenser lenses interposed between the light source and the eye. A spectrophotometric comparison of the energy distributions of the 1500-watt lamps and the pointolite both run at 100 volts, with a standard lamp of known colour temperature showed that these energy distributions corresponded approximately to 2800° K. The relative energy distribution in our white light is plotted in fig. 3, curve I, where the value at $\lambda = 0 \cdot 59\ \mu$ is taken as unity.

Coloured conditioning stimulations were obtained by interposing liquid colour filters in the white beam at T. The spectral transmissions of these filters were measured and by multiplying the spectral transmission by the relative energy value for white at each wave-length, the relative energy distribution in the coloured light is obtained. The four colour filters used in this work were designed to transmit limited ranges of the spectrum in the blue (filter A′), blue green (filter B′), yellow (filter D), and red (filter F) respectively. The corresponding relative energy distributions derived by the method just indicated are shown in curves II to V of fig. 3.

Knowing the relative energy distribution, the specification of the stimulation is completed by measuring the brightness by normal photometric methods.

When the surround was white, its brightness was determined by direct measurement with an illuminometer set up at the aperture through which the subject viewed the field. For low intensities, the brightness was determined

from a measurement at high value and the known transmissions of neutral filters used to obtain the low intensity.

The intensity of the glare source is most usefully expressed for the present

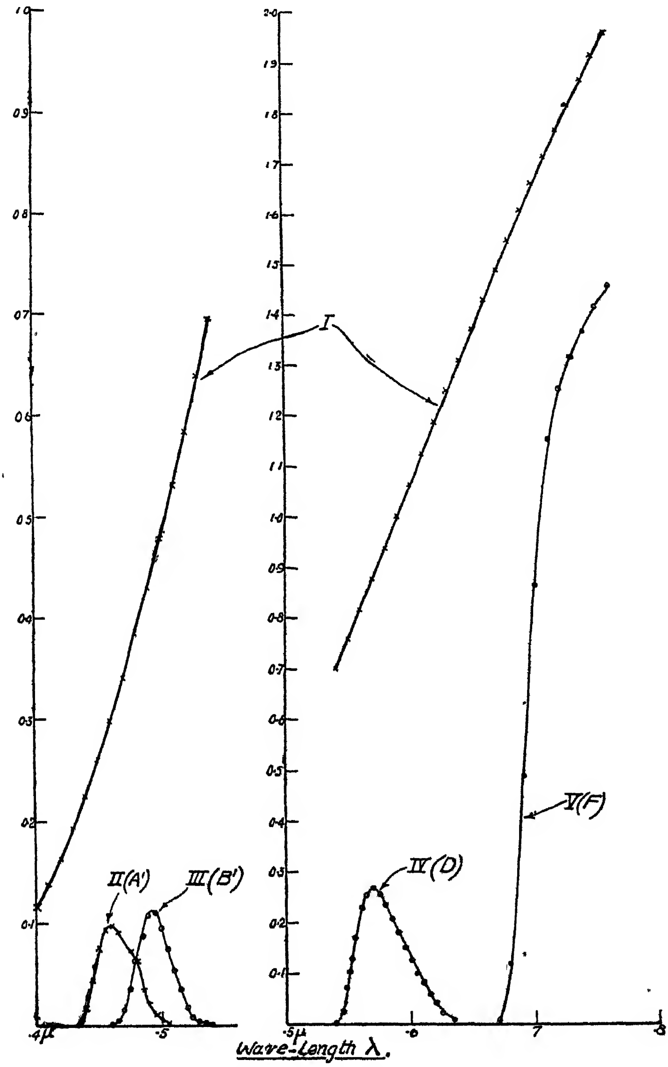


FIG. 3.—Relative energy distribution for white and coloured conditioning stimulations.

purpose, by giving the illumination E produced on a vertical plane in the position of the subject's pupil. This measurement was made by setting up an opal glass in the desired plane and observing its brightness with an illuminometer through the aperture Z. Calibration was effected as usual by illuminating

the opal with a standard lamp of known candle power and obtaining a brightness measurement under these conditions.

When the brightness to be measured is not white, the ordinary equality of brightness illuminometer gives a measurement of very low precision. To deal with such light the illuminometer was adapted to employ the flicker method. With its aid the brightness B_s was determined for colours in the same way as for white. The values of B_s obtained by different observers by the flicker method (or any other method of visual heterochromatic photometry) differ somewhat and particularly for blues and reds of high saturation, owing to differences in the observers' luminosity curves. For example, the ratio of the brightnesses as measured by W. S. S. and B. H. C. respectively for various coloured lights including the four used here, was found to be as follows :—

	Blue-violet (filter A).	Blue (filter A′).	Blue-green (filter B′).	Green (filter C).
$\frac{\text{B (W.S.S.)}}{\text{B (B.H.C.)}}$	1·87	1·37	1·06	0·92

	Yellow (filter D).	Pale red (filter E).	Red (filter F).
$\frac{\text{B (W.S.S.)}}{\text{B (B.H.C.)}}$	0·95	1·08	1·28

Thus there is still a certain measure of indefiniteness in our specification of the conditioning stimulation for colours. The values of B and B_s for colours used here were measured by B. H. C.

As we have used an artificial pupil instead of the natural pupil we must indicate this in stating our conditioning stimulation, *e.g.*, white surround of 300 candles/sq. ft. seen through a 2 mm. diameter artificial pupil.

Turning now to the test stimulus, to obtain a monochromatic test spot use was made of the principles of Maxwellian view. The entrance slit X_1, fig. 2, of a constant deviation Hilger spectrometer was illuminated by means of a ground glass plate J behind which was placed a projection lamp (100 watt) K. The exit slit X_2 of the spectrometer delimits a narrow band of the spectrum and an image of this band was focussed on to the subject's eye at O by means of the lens L_1, the light being reflected by the prism P and passing through the central aperture in the background screen. A second lens L_2 placed close to the exit slit of the spectrometer caused an image of the aperture in a diaphragm inserted at F, to be formed in the plane of the background screen in the middle of the aperture H. This image was seen sharply in focus by the subject, and constituted the test spot. The image of the exit slit of the spectrometer was

adjusted to fall in the centre of the artificial pupil at O and was delimited by the latter in the vertical direction, as shown in fig. 4. In the later work a larger (3 mm. diameter) artificial pupil was used and the exit slit of the spectrometer was itself delimited in the vertical direction by suitable diaphragms. The adjustment then consisted in bringing the exit slit image into the middle of the artificial pupil, taking care that no part of it was cut off. The intensity of the test spot was varied by slowly changing the voltage applied to the projection lamp. This was done automatically by means of a potentiometer, the slider of which was pulled along by a wire passing round a motor driven pulley wheel. With constant motor speed, the test spot intensity increased or decreased by an approximately constant proportion in unit time. When the subject decided the critical intensity had been reached he operated a switch which instantly stopped the motor driving the potentiometer slider. The experimenter then read the voltage acting across the contacts of the projection lamp.

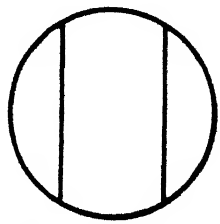


FIG. 4. — Image of spectrometer exit slit in the artificial pupil.

Instead of expressing the test spot intensity by giving the photometric brightness determined by heterochromatic photometry (*e.g.*, flicker photometry), it was decided to adopt the more fundamental method of giving the brightness in energy units. Let us suppose that the dimensions of the entrance and exit slits of the spectrometer, the positions of the ground glass and the projection lamp are all kept constant. For a given voltage v applied to the projection lamp, the radiation emitted by the filaments will have a certain spectral distribution which we may write as $E_\lambda(v)$. The optical parts interposed between the filaments of the projection lamp and the eye of the subject will together have a certain transmission factor t_λ which varies with λ. Now, owing to the different dispersion of the spectrometer in different parts of the spectrum, the width $\Delta\lambda$ of the narrow spectral band isolated by the fixed entrance and exit slits of the spectrometer, will vary with wave-length. We may write $\Delta\lambda = p_\lambda \, \Delta\lambda_{0 \cdot 59}$, where $\Delta\lambda_{0 \cdot 59}$ is the width at $\lambda = 0 \cdot 59$ and p_λ is a function which can easily be determined experimentally by known methods.

Thus the flux of energy from the test spot entering the subject's eye will be proportional to

$$A \times E_\lambda(v) \times t_\lambda \times p_\lambda \times \Delta\lambda_{0 \cdot 59}, \tag{1}$$

where A is the area of the test spot.

The transmission t_λ varies with wave-length principally because of selective

absorption in the spectrometer. We have assumed that apart from the latter, the optical parts transmit equally in all regions of the spectrum. The transmission of the spectrometer (which we now put equal to t_λ) at different wave-lengths was determined directly with the aid of a second spectrometer and this determination is briefly described in the Appendix, where a table showing the results is also given. When working with a test spot in the extreme blue or red there is some danger of scattered light of wave-length near the centre of the spectrum where the visibility is high vitiating the measurements. This was avoided by inserting in the test spot beam a blue or red glass filter with high transmission in the blue or red and very low transmission elsewhere. The transmissions b_λ and r_λ of these stray light glasses were determined and allowance was made where necessary in calculating the test spot intensity.

It is known that the energy distribution of the radiation from incandescent tungsten approximates closely to the energy distribution from a black body appropriate to some temperature. Suppose that this temperature has the value T_v when the voltage applied to the projection lamp is v. Then the distribution of energy in the spectrum $E_\lambda(v)$ may be written by Wien's radiation law (which is accurate in the temperature wave-length range here considered):

$$E_\lambda(v) = \text{const. } \lambda^{-5} \, . \, e^{-C_2/\lambda T_v}. \tag{2}$$

Suppose that we know T_v when $v = 100$ volts. $E_\lambda(100)$ can then be obtained from tabulated values of the expression (2). For any other voltage v we have

$$\log \frac{E_\lambda(v)}{E_\lambda(100)} = -\frac{C_2}{\lambda}\left\{\frac{1}{T_v} - \frac{1}{T_{100}}\right\} = -D_{\lambda v}. \tag{3}$$

If we take a particular wave-length λ_0 we can determine

$$\log \frac{E_{\lambda_0}(v)}{E_{\lambda_0}(100)} = -D_{\lambda_0 v}$$

as a function of the voltage v, by a spectrophotometric method. Thus for this wave-length λ_0, we are able to write down $\log E_{\lambda_0}(v)$ for any voltage v:

$$\log E_{\lambda_0}(v) = \log E_{\lambda_0}(100) - D_{\lambda_0 v}. \tag{4}$$

(from tables) (Experimental calibration)

For any other wave-length λ, it follows from (3) that

$$\log E_\lambda(v) = \log E_\lambda(100) - \frac{\lambda_0}{\lambda} D_{\lambda_0 v}. \tag{5}$$

It is clear, therefore, that the relative energy distribution in the spectrum for any voltage is determinable from two experiments :—

(1) Determination of T_{100}

(2) Determination of the values of $\log \dfrac{E_{\lambda_0}(v)}{E_{\lambda_0}(100)}$ for a particular wave-length λ_0.*

The value of T_{100} for the projection lamp used was determined by measuring with a spectrophotometer the energy of the projection lamp relative to that of a standard lamp of known colour temperature (2360° K.), for different wave-lengths throughout the spectrum. The energy distribution of the projection lamp having been derived in this way, the temperature corresponding to the black body energy distribution which fitted best the experimental values was determined. This temperature was found to be $T_{100} = 2720°$ K.

Taking $\lambda_0 = 0 \cdot 56$, a careful determination was made of $D_{\lambda_0 v}$ as a function of voltage, from $v = 28$ to $v = 100$ volts. Additional measurements were made at other wave-lengths to test the validity of the equation (5). The results were in very satisfactory agreement with the theory.

Collecting together the various factors determining the test spot intensity, we may say that if $I_\lambda(v)$ is the flux of energy in ergs./sec. from the test spot, falling on the subject's eye, then

$$\log I_\lambda(v) = \log p_\lambda + \log t_\lambda + \frac{\log b_\lambda}{\log r_\lambda} + \log \underline{E}_\lambda(100)$$
$$- \frac{0 \cdot 56}{\lambda} D_{0 \cdot 56 v} + \log A + \log \Delta\lambda_0 + \text{const.}, \qquad (6)$$

where the constant is independent of wave-length and lamp voltage.

For studying the relative l.b.i.'s for different wave-lengths the constant in the above equation is unimportant. To obtain an estimate of the absolute value of the constant the following procedure was adopted. For the wave-length $\lambda = 0 \cdot 59\ \mu$ (yellow), the spot intensity was adjusted until it matched in brightness a white surround of fixed known brightness as seen by the subject with his eye applied to the 2 mm. artificial pupil. For this purpose the test spot aperture was removed so that monochromatic light filled the whole of the $1 \cdot 28°$ diameter centre field. Suppose the brightness of the white surround is B_{s_0} candles/sq. ft. Then from unit area of the surround the subject's eye

* This method was worked out by Buckley and Brookes, 'J. Sci. Instr.,' vol. 7, p. 305 (1930).

receives $B_{s_0} \times \delta/r^2$ lumens of white light where δ is the area of the artificial pupil and r is the distance from the surround to the eye. If the monochromatic centre field is adjusted to match the surround in brightness, then the eye also receives per unit area of the centre field the same number of lumens, of light of wave-length $\lambda = 0 \cdot 59\ \mu$.

The various investigations which have been made to determine the luminosity function and the mechanical equivalent of light, have been summarized by Hyde (1921). Hyde finds that for light of wave-length corresponding to the maximum of the luminosity curve, the mechanical equivalent of light M, based on direct comparison photometry, is given by

$$M = 0 \cdot 00150 \text{ watts/lumen} = 15000 \text{ ergs./lumen sec.}$$

For any other wave-length λ the mechanical equivalent is obtained by dividing M by the relative luminosity value V_λ. Thus $B_{s_0}\delta/r^2$ lumens of yellow light ($\lambda = 0 \cdot 59$), represent

$$\frac{B_{s_0} \times \delta}{r^2} \times \frac{M}{V_{0 \cdot 59}} \text{ ergs./sec.}$$

$$= \frac{B_{s_0} \times \delta}{r^2} \times \frac{15000}{0 \cdot 74} \text{ ergs./sec.}$$

This is only true, however, if the subject who makes the match between the white surround and the monochromatic centre field, has a luminosity curve which agrees exactly with the mean luminosity curve which was adopted by Hyde and which is involved in the computation of M. No such subject exists, but by using a large number of subjects and taking the mean of their settings a sufficient approximation is obtained to the correct result.

The total energy flux from the whole of the centre field is clearly given by

$$A' \frac{B_{s_0} \times \delta}{r^2} \times \frac{15000}{0 \cdot 74} \text{ ergs./sec.},$$

where A' is the area of the centre field, and this must equal $I_{0 \cdot 59}(v_0)$, where v_0 is the voltage at which the centre field matched the white surround. We have, using equation (6),

$$\log A' + \log B_{s_0} + \log \frac{\delta}{r^2} \frac{15000}{0 \cdot 74},$$

$$= \log I_{0 \cdot 59}(v_0),$$

$$= \log p_{0 \cdot 59} + \log t_{0 \cdot 59} + \log E_{0 \cdot 59}(100) - \frac{0 \cdot 56}{0 \cdot 59} D_{0 \cdot 56}{}^{v_0},$$

$$+ \log A' + (\log \Delta\lambda_0 + \text{const.}). \qquad (7)$$

The only unknown is the expression ($\log \Delta\lambda_0 +$ const.), the value of which is therefore determined by this equation. Knowing the value of the calibration constant $C = \log(\Delta\lambda_0 + \text{const.})$, it is clear that $\log I_\lambda(v)$ is determinable for any voltage v, wave-length λ, and test spot area A.

A redetermination of C was necessary whenever the sizes of the entrance or exit slits of the spectrometer were altered or when the position of the ground glass with respect to the projection lamp was changed. The original determination of the calibration constant C was based on measurements by 24 subjects. In subsequent recalibrations only one or two subjects, were employed. These subjects (W. S. S. and B. H. C.) were known to give results differing very little from the mean results, and a correction was made for the small difference involved.

The coloured test spot is not strictly monochromatic because in reality a small band of the spectrum is being used. The energy distribution in this band has approximately the shape shown in fig. 5, if the entrance and exit

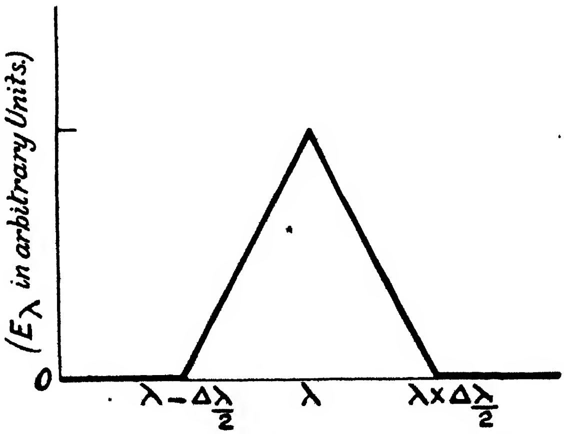


FIG. 5.—Relative energy distribution in narrow spectral band used for test stimulus.

slits of the spectrometer are of the same width, which was always so. The wave-length difference $\Delta\lambda$ between the extreme limits of the band is taken as defining the purity. As stated above, with fixed spectrometer slits $\Delta\lambda$ varies with position in the spectrum ($\Delta\lambda = p_\lambda \Delta\lambda_{0\cdot59}$). In the first series of measurements the spectrometer slits were of a width to give $\Delta\lambda_{0\cdot59}$ = approximately $0\cdot0090\ \mu$. Thus at the blue end ($\lambda = 0\cdot40$) $\Delta\lambda$ equalled $0\cdot0021\ \mu$ and at the red end ($\lambda = 0\cdot76$) $\Delta\lambda$ equalled $0\cdot0195\ \mu$. In the later measurements the slits were changed in different parts of the spectrum in order to give a band width not exceeding $0\cdot01\ \mu$ throughout the spectrum.

All the measurements were obtained using a rectangular test spot of 0·70° horizontal, 0·16° vertical side. Suppose that for a particular wave-length λ, the voltage v corresponding to liminal vision of the test spot is v_t. The total flux of energy in ergs./sec. collected by the subject's eye is then $\mathrm{I}(v_t)$. The *angular area* of the test spot, a is clearly given by $a = 0{\cdot}70 \times 0{\cdot}16$ in degree units. We shall define the liminal brightness increment in energy units as

$$\mathrm{U}_\lambda = \mathrm{I}_\lambda (v_t)/a.$$

Thus, in words, U_λ is the energy flux in ergs./sec. received by the eye per unit angular area of the test spot. It is convenient to use a logarithmic scale when plotting U_λ and we shall actually plot the logarithm of the reciprocal of the l.b.i., *i.e.*, log $1/\mathrm{U}_\lambda$, in anticipation of later developments.

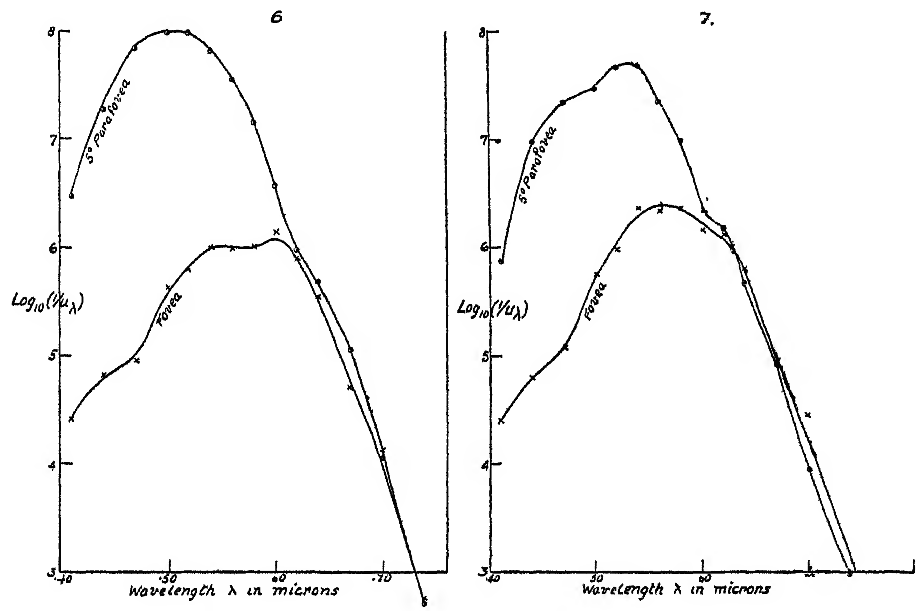
In a given run, two determinations of the voltage v_t were made at each wave-length, corresponding respectively to the disappearance and appearance l.b.i.'s. The value of log $1/\mathrm{U}_\lambda$ was derived from the mean of the two voltage readings. Thus each such value is based on no more than two observations. The necessity for completing a run within a reasonable period (1·5 to 3 hours) made it impossible to take several pairs of readings at each wave-length with the object of improving the precision. Other work has indicated that it is definitely more advantageous to cover the whole range of wave-length in a single run, even at the loss of precision, than to make more precise measurements on portions of the range in separate runs and then attempt to link up the results.

4. *The Measurements for White Conditioning Stimulations.*

Conditioning Stimulation I.—We shall consider first the results obtained in concurrent runs for foveal and 5°-parafoveal vision with the totally dark-adapted eye, figs. 6 and 7. The subject was kept in total darkness for a period of 1 hour prior to the commencement of the measurements. During the run the visual field was completely dark except for the test spot and a very weak point source of light (adjustable to be just visible) 5° from the test spot which served as fixation or orientation point. Foveal and parafoveal readings were taken alternately. For parafoveal readings the test spot was exposed one second every 4 seconds. For the foveal readings the test spot was exposed continuously. As the fovea was a region of minimum sensitivity for both subjects under the conditions of these measurements, the foveal reading corresponded to an intensity of the test spot when by no slight readjustment of the direction of vision the test spot could be made to disappear (appearance

threshold). By making use of this criterion the difficulty of foveal fixation was in some degree overcome.

It is clear from figs. 6 and 7 that for both subjects the parafoveal l.b.i. value greatly exceeds the foveal value for wave-lengths less than about 0·62 μ. The curves resemble in general character those obtained for one group of subjects by Abney and Watson (1916) and we may conclude that the subjects W. S. S. and B. H. C. belong to Abney and Watson's class I, subjects with rod-free fovea. Other comments on these curves are postponed until later.



FIGS. 6 and 7.—Log $1/U_\lambda$ against λ for dark-adapted eye. Single runs. Conditioning stimulation I. Fig. 6, subject W. S. S.; fig. 7, subject B. H. C.

Conditioning Stimulation II. White Surround of High Brightness.—Passing from the totally dark adapted-eye to the other extreme, it was desired to determine the l.b.i. as a function of wave-length when the retina is adapted to a very high brightness level. A uniform surround brightness of white light B_s of approximately 300 candles/sq. ft. was taken as conditioning stimulation, the circular centre field of 1·28° diameter being kept at zero brightness. The rectangular test spot appeared in the middle of the dark centre field. During the period of actual observation, *i.e.*, while the voltage on the projection lamp was steadily increased (or decreased) until the test spot became visible (or invisible), the subject contrived, as far as possible, to keep his direction of vision

unchanged so that the dark centre field fell on the same patch of retina. To this extent, therefore, the measurements correspond to an unstimulated area of the retina in equilibrium with a highly stimulated surround. Between actual observations the subject relaxed his attention and his eye was allowed to wander.

The variation of $\log(1/U_\lambda)$ with λ obtained under these conditions was of an unexpected character and several independent runs were made. The values of $\log(1/U_\lambda)$ obtained in different runs under similar conditions, exhibit wide variations although the curve of $\log(1/U_\lambda)$ against λ shows in every case to a greater or lesser degree the salient features present in the mean curve. We give in fig. 8 two examples of the results of single runs and in fig. 9 the results of all

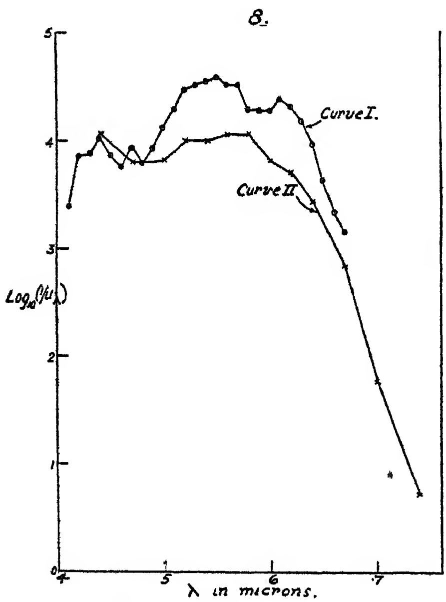


FIG. 8.—$\log 1/U_\lambda$ against λ. Single runs. Conditioning stimulation II. $B_s = 300$ candles/sq. ft. white (2 mm. diameter artificial pupil). Curve I, B. H. C. fovea; curve II, B. H. C. 5°-parafovea.

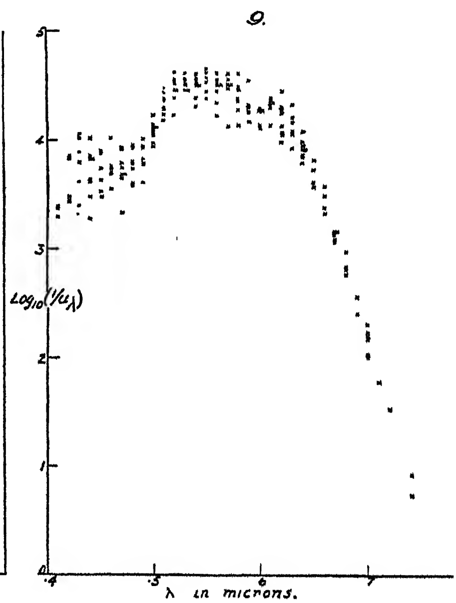


FIG. 9.—$\log 1/U_\lambda$ against λ. Collected results for B. H. C. fovea. Conditioning stimulation II. $B_s = 300$ candles/sq. ft. white (2 or 3 mm. diameter artificial pupil).

runs for B. H. C. fovea. The variations in different runs are due mainly to fluctuations in the subject's l.b.i. values but possibly in part to minor modifications in the experimental method (*e.g.*, the spectral purity in the red end of the spectrum was improved in the later runs and the surround brightness was not always exactly 300 candles/sq. ft.).

We shall base our discussion of the results on the mean data given in figs. 10 and 11. Considering first the foveal results it is clear that the curves show:

(*a*) A well-defined maximum at $\lambda = 0{\cdot}536$ (W. S. S.), $\lambda = 0{\cdot}540$ (B. H. C.).

(*b*) A second maximum at $\lambda = 0{\cdot}434$ (B. H. C.), $\lambda = 0{\cdot}446$ (W. S. S.).

(*c*) A weak third maximum at $\lambda = 0{\cdot}602$ for W. S. S. and for B. H. C. a tendency to form a maximum at about the same wave-length.

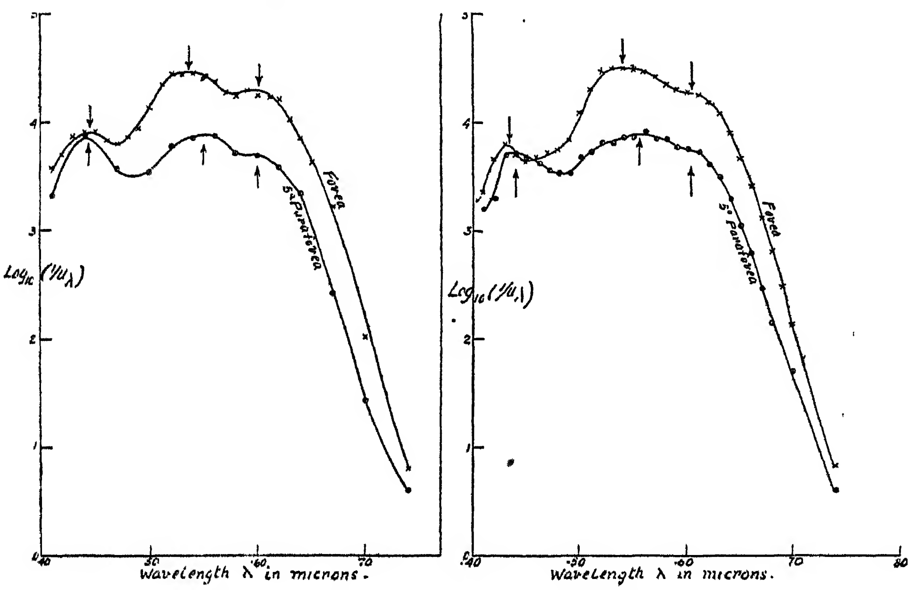
The parafoveal curves (based on fewer runs) show:—

(*a*) A maximum at $\lambda = 0{\cdot}554$ (B. H. C.), $0{\cdot}550$ (W. S. S.).

(*b*) A second maximum at $\lambda = 0{\cdot}440$ (B. H. C.), $0{\cdot}444$ (W. S. S.).

(*c*) A tendency to form a maximum at $\lambda = 0{\cdot}60$ μ.

10. 11.



FIGS. 10 and 11.—Log $1/U_\lambda$ against λ. Mean data for foveal and 5°-parafoveal vision. Conditioning stimulation II. $B_s = 300$ candles/sq. ft. white (2 or 3 mm. diameter artificial pupil). Fig. 10, Subject W. S. S.; fig. 11, subject B. H. C.

In giving exact values of the wave-lengths at which the curves as drawn exhibit maxima, it is not, of course, suggested that the data fix these wave-lengths to the accuracy given. The l.b.i. measurements are necessarily crude and the derivations of wave-lengths for maxima are correspondingly approximate.

There is a particular law of variation of $1/U_\lambda$ with λ which might with reason be expected to apply for the light-adapted eye. Different coloured lights at

their threshold intensities may be regarded in a certain sense, as of equal brightness. Thus multiplying the intensity U_λ by the luminosity (visibility) factor V_λ we should obtain a constant. In other words $1/U_\lambda$ should be proportional to V_λ. The luminosity function V_λ for the light-adapted eye has been determined by König, Abney and a number of American investigators and a representative mean curve has been adopted by the International Commission on Illumination.* In fig. 12 we have plotted log V_λ against λ, using

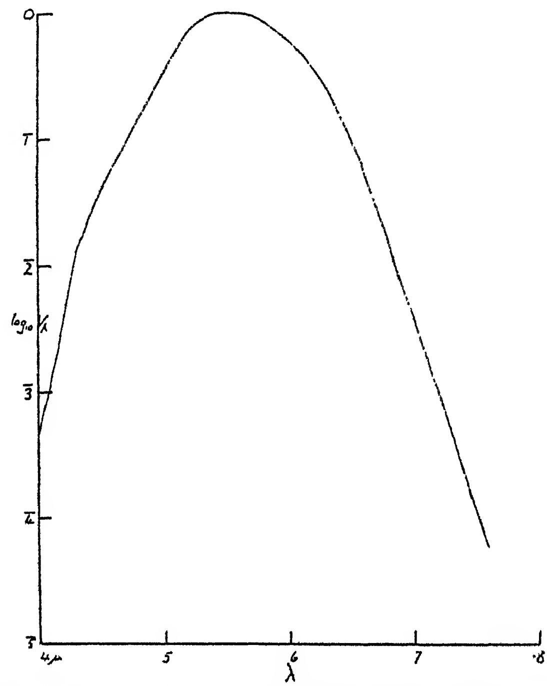


FIG. 12.—Log V_λ against λ. V_λ is the luminosity function, I.C.I. revised values, 1924.

the I.C.I. values. Comparing the log V_λ curve with the foveal curves of figs. 10 and 11, it is clear that if the log V_λ curve is adjusted, by displacement parallel to the ordinate axis, to fit the log $1/U_\lambda$ curve as nearly as possible in the range $\lambda = 0 \cdot 50$ to $0 \cdot 70$, then very large differences between the curves are apparent in the range $\lambda < 0 \cdot 46\ \mu$, where $1/U_\lambda$ may exceed V_λ by more than 10 times. Even in the range $0 \cdot 50$ to $0 \cdot 70$ a good fit between the log V_λ and log $1/U_\lambda$ curves is not possible although the discrepancies here are not so marked.

A possible practical repercussion of these results is that in evaluating the

* 'Proc. International Commission on Illumination' (1924).

luminosities of coloured signals for use in daylight the I.C.I. visibility curve may have to be replaced by a curve resembling those shown in figs. 10 and 11.

We shall now give the log $(1/U_\lambda)$ curves obtained in single runs with other conditioning stimulations.

Conditioning Stimulation III and IV. White Glare Source at 3° above Test Spot.—Fig. 13 shows the foveal and 5°-parafoveal curves of log $1/U_\lambda$ against

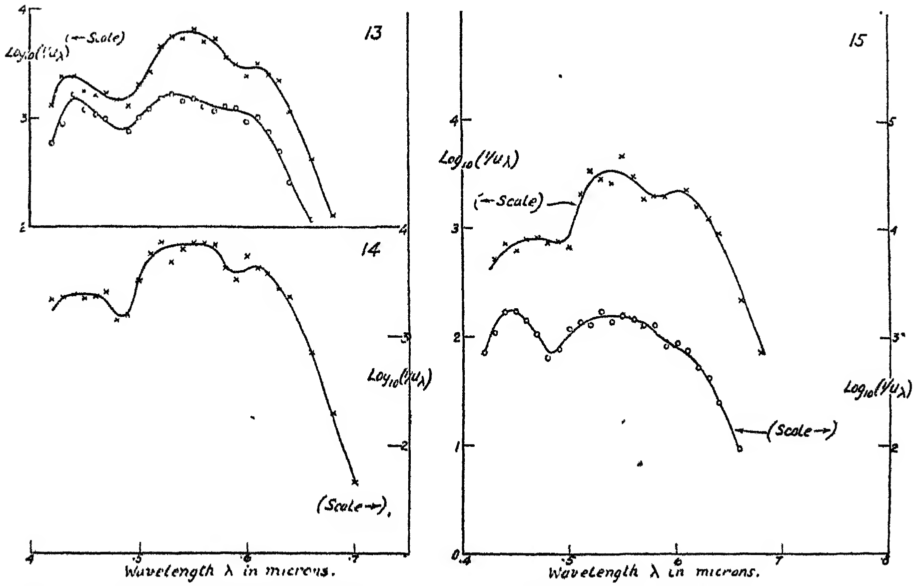


FIG. 13.—Log $1/U_\lambda$ against λ. Single runs, B. H. C. fovea and 5°-parafovea. Conditioning stimulation III. E = 57 foot-candles white (3 mm. diameter artificial pupil). B. H. C.; ×, fovea; ○, 5°-parafovea. FIG. 14.—Log $1/U_\lambda$ against λ. Single run, B. H. C. fovea. Conditioning stimulation IV. E = 57 foot-candles white. B_s = 300 candles/sq. ft. white (2 mm. diameter artificial pupil). B. H. C., fovea. FIG. 15.—Log $1/U_\lambda$ against λ. Single runs, B. H. C. fovea and 5°-parafovea. Conditioning stimulation V. B_s = 70 candles/sq. ft. white; B = 100 candles/sq. ft. (foveal run), B = 70 candles/sq. ft. (parafoveal run); (3 mm. diameter artificial pupil). B. H. C.; ×, fovea; ○, 5°-parafovea.

λ obtained in single runs. The glare source was the image of a pointolite plate and the vertical illumination at the eye equalled 57 foot-candles. Both curves show well-marked maxima in the blue and green and a rudimentary maximum at about 0·60 μ. For these measurements, apart from the glare source the field was completely dark. Fig. 14 shows a set of foveal readings with a surround brightness of 300 candles/sq. ft. in addition to the glare source. Again the data are consistent with a curve exhibiting three maxima.

Conditioning Stimulation V. High Brightness White Centre Field and White Surround.—In all the previous conditioning stimulations the test spot appeared on a retinal area whose direct stimulation was otherwise zero. We now have a white brightness in the centre field in addition to a white surround. The results of a foveal and a 5°-parafoveal run obtained in this way with centre field brightnesses of 100 and 70 candles/sq. ft. respectively and a surround brightness of 70 candles/sq. ft. are shown in fig. 15. We see that the curves exhibit the same general character as obtained with a bright surround or an intense glare source. For the foveal curve the maximum in the blue end of the spectrum appears to be displaced towards the red compared with the previous curves, but very little weight can be attached to this peculiarity occurring in a single run in which in any case the maximum is only very weakly defined.

Summing up all the observations made with an intense conditioning stimulation—surround, glare or background—we may say that for each the log $(1/U_\lambda)$ curve for foveal vision possesses three maxima, or two maxima and a rudimentary maximum, whose positions are given approximately by $\lambda = 0\cdot44\ \mu$, $\lambda = 0\cdot54\ \mu$, $\lambda = 0\cdot60\ \mu$. For 5°-parafoveal vision the same remark applies except that, as the centre maximum is very flat, its position is correspondingly uncertain.

As the brightness level is decreased the log $(1/U_\lambda)$ curves must change shape in continuous fashion and must reduce finally to the curves for zero conditioning stimulation. Several runs were made with surround brightnesses B_s corresponding to intermediate conditioning stimulations. Fig. 16 shows a foveal run with $B_s = 8$ candles/sq. ft. The maximum in the blue has sunk with respect to the central maximum although it is still definite. The rudimentary maximum in the red shows a slight rise with respect to the central maximum. In fig. 17 foveal and 5°-parafoveal curves for $B_s = 0\cdot30$ candles/sq. ft. are reproduced. For the fovea the maximum in the blue has now degenerated to no more than a slight swelling on the left-hand slope of the central maximum. The rudimentary maximum in the red appears the same as for $B_s = 8$ candles/sq. ft. For the parafovea, the blue maximum is much weakened compared with the high brightness condition and this time the maximum in the red is slightly depressed compared to the central maximum. Similar remarks apply to the parafoveal curve of fig. 18 which refers to subject W. S. S. The blue maximum in this curve is still clearly defined and this is consistent with our experience that the maxima are in general more pronounced for subject W. S. S. than for subject B. H. C.

Reverting to figs. 6 and 7 giving log $(1/U_\lambda)$ curves for zero stimulation, we see that for the fovea the blue maximum is reduced to a mere bulge. As regards the maximum in the red the curves for the two subjects give different indications, B. H. C. shows a depression W. S. S. a rise with respect to the central maximum. The figures give the data for single runs only and too much weight must not be attached to the results.

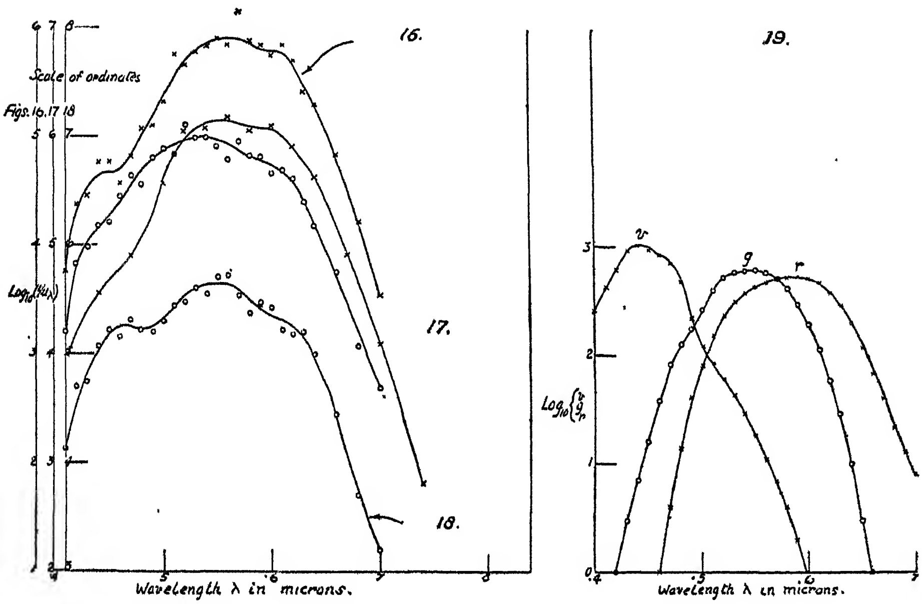


Fig. 16.—Log $1/U_\lambda$ against λ. Single run, B. H. C. fovea. Conditioning stimulation II. $B_s = 8$ candles/sq. ft. white (2 mm. diameter artificial pupil). Fig. 17.—Log $1/U_\lambda$ against λ. Single runs, B. H. C. fovea and 5°-parafovea. Conditioning stimulation II. $B_s = 0{\cdot}3$ candles/sq. ft. white (2 mm. diameter artificial pupil). ×, fovea; O, 5°-parafovea. Fig. 18.—Log $1/U_\lambda$ against λ. Single run, W. S. S. 5°-parafovea. Conditioning stimulation as for fig. 17. Fig. 19.—König-Dieterici-Abney-Weaver trichromatic excitation curves *v*, *g*, *r*, for an equi-energy spectrum, plotted to a logarithmic scale.

5. *Discussion of the Results.*

The above study of the effect of brightness level on the log $(1/U_\lambda)$ curve together with the results for coloured conditioning stimulations to be given later have suggested the following hypothesis.

For any given condition of the foveal retina, determined by specifying the

conditioning stimulation, the reciprocal l.b.i. $1/U_\lambda$ is a function of wave-length λ of the form :

$$1/U_\lambda = \eta_1 X_1(\lambda) + \eta_2 X_2(\lambda) + \eta_3 X_3(\lambda), \tag{8}$$

where $X_1(\lambda)$, $X_2(\lambda)$ and $X_3(\lambda)$ are fixed functions of λ independent of the conditioning stimulation and η_1, η_2, η_3 are three parameters independent of λ but dependent on the conditioning stimulation. In addition, each of the three fixed functions exhibits a single maximum,

X_1 at λ_1 which is in the near neighbourhood of $0 \cdot 44$ μ,
X_2 at λ_2 which is in the near neighbourhood of $0 \cdot 54$ μ,
X_3 at λ_3 which probably lies slightly to the long-wave side of $0 \cdot 60$ μ (at $0 \cdot 62$ μ possibly).

The wave-lengths at which maxima occur for $1/U_\lambda$ or $\log (1/U_\lambda)$ as given by equation (8) will not in general coincide with λ_1, λ_2, λ_3, and, in fact, their positions will vary with the relative values of η_1, η_2, η_3 in any particular case. The above tentative estimate of the values of λ_1, λ_2, λ_3 is, however, based on the position of the maxima in the experimental $\log (1/U_\lambda)$ curves, observing that where a maximum is well defined, its displacement from a maximum of a fixed function is likely to be small. Referring to the analysis of retinal adaptation put forward in a recent paper (Stiles and Crawford, 1932), we recall that if the state of the retina requires n variables or "adaptation potentials" ξ_1, ξ_2 ..., ξ_n, for its specification, then the result of any visual test is a function of the form

$$\sigma = f(\mu;\ \xi_1, \ldots, \xi_n),$$

in which the effect of the conditioning stimulation can enter only through the values of $\xi_1, \ldots, \xi_n$, the symbol μ representing all the variables concerned with the test stimulation. The expression for $1/U_\lambda$ given in (8) represents a special case of this relation in which the conditioning stimulation acts by determining the values of *three* parameters η_1, η_2, η_3. We cannot, however, assert that three adaptation potentials ξ_1, ξ_2, ξ_3 suffice to define the state of the retina, and identify ξ_1, ξ_2, ξ_3 with η_1, η_2, η_3 because the adaptation potentials must represent the effect of conditioning stimulation in the expressions for the numerical results of *all* tests and we have studied only the limited group of tests formed by varying the wave-length of the monochromatic test spot in a l.b.i. determination. Nevertheless, within this limited group of tests η_1, η_2, η_3 play the part of adaptation potentials and provide a simple illustration of the theory. If later work shows that *three* adaptation potentials ξ_1, ξ_2, ξ_3 are sufficient, then a set of values of η_1, η_2, η_3 will determine the values of ξ_1, ξ_2, ξ_3 and conversely.

If we select from among all possible conditioning stimulations, those for which the brightnesses in the field are all white, we have evidence (namely, the equivalent background principle (Stiles and Crawford, 1932)) for thinking the retinal condition specifiable by one adaptation potential (or level). For white conditioning stimulations, therefore, we may expect η_1, η_2 and η_3 all to be determined given one of them or, expressed in another way, η_1, η_2 and η_3 will each be a function of a single adaptation level which can be taken to be the equivalent background brightness β : $\eta_1 = r_1(\beta)$, $\eta_2 = r_2(\beta)$, $\eta_3 = r_3(\beta)$.

Logically, the next step would be to use the data of figs. 6–18 to obtain approximate numerical specification of the functions X_1, X_2, X_3 and to determine η_1, η_2, η_3 as functions of the conditioning stimulation. Work on these lines is proceeding, but it will probably be necessary to make further measurements before even a first approximation can be obtained.

The close relationship of the above considerations with the trichromatic theory of vision is at once apparent. The central experimental fact around which the trichromatic theory is built is the three-dimensional character of colour matching equations. This condition would be consistent with the existence of three kinds of receptors, each kind having a specific excitation curve, but the colour mixture data do not of themselves uniquely determine these excitation curves. By introducing auxiliary considerations, various sets of three excitation curves have been derived notably the König-Dieterici " Elementarempfindungen " and the " Grundempfindungen " and the Hecht (1932) cone excitation curves. The König-Dieterici elementarempfindungen as modified by Weaver (1922) to take account of measurements by Abney each show a single maximum, at wave-lengths $0 \cdot 44\ \mu$, $0 \cdot 55\ \mu$ and $0 \cdot 59\ \mu$ respectively. The peaks of the Hecht curves, on the other hand, all lie in the range $0 \cdot 54\ \mu$ to $0 \cdot 57\ \mu$. The evidence for either set is indirect and has been criticized in various quarters.

If we are correct in associating the three functions X_1, X_2, and X_3 with the three mechanisms which are responsible for the three-dimensional character of colour matching equations, then there must be a close relation between the curves X_1, X_2, X_3 and the excitation curves derived from colour mixture data. It is not necessary that X_1, X_2, X_3 should coincide exactly with some set of colour mixture excitation curves, although this may be the case. The great advantage of the present method of approach over derivations from colour mixture data is that there can be no question of changing the wave-lengths of maximum sensitivity of the three mechanisms by a mathematical transformation. Even with the comparatively meagre data available for determining

the curves X_1, X_2, X_3 certain conclusions can be drawn: (1) three receptor mechanisms with maximum sensitivities very close together in the middle of the visible spectrum as required in Hecht's theory are definitely ruled out; (2) however questionable the arguments previously advanced for the elementarempfindungen or grundempfindungen may be, the wave-lengths of maximum sensitivity of the blue and green curves are very close to the wave-lengths of the maxima of the curves X_1 and X_2. The maximum in the red curve occurs at $0 \cdot 59\ \mu$ (elementarempfindungen) and $0 \cdot 56\ \mu$ (grundempfindungen) but our data fail to determine the maximum in X_3 sufficiently well to justify comparison. Fig. 19 shows the König-Dieterici-Weaver excitation curves plotted to a log scale for comparison with our data for $\log (1/U_\lambda)$.

So much for foveal vision. For 5°-parafoveal vision, an instructive approach to the results is obtained by plotting against wave-length the ratio of the parafoveal to the foveal reciprocal l.b.i. or preferably the logarithm of this ratio, $\log (1/U_\lambda)_{\text{parafoveal}} - \log (1/U_\lambda)_{\text{foveal}} \equiv \Delta$. This is done for surround brightnesses $B_s = 300, 0 \cdot 3$ and 0 candles/sq. ft. in fig. 20 and for a high background brightness and an intense glare stimulation in fig. 21. The outstanding feature of all the curves is the appearance of a more or less pronounced maximum in the blue end of the spectrum. Reasons which may be put forward to account for the difference between foveal and parafoveal sensitivities are the following:

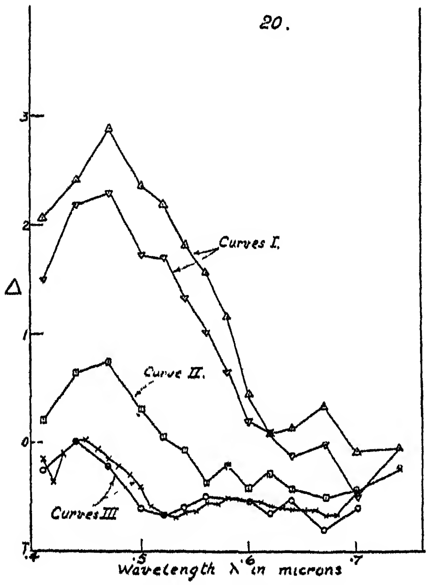
(1) The parafovea may give a lower value of $(1/U_\lambda)$ than the fovea because image formation is less good and the retinal structure is coarser in the parafovea. A lowering of $(1/U_\lambda)$ due to causes of this nature may be expected to affect the whole of the spectrum.
(2) Yellow pigment is present at the fovea and not at the parafovea, so that the latter will be relatively more sensitive in the blue end.
(3) At low brightness levels the parafovea is served by the rods, the fovea by the cones, and the sensitivity of the rods greatly exceeds that of the cones in the blue end.

Cause (1) is no doubt operative at high brightness levels and accounts for the negative values for Δ in figs. 20 and 21. With regard to causes (2) and (3), the former may be claimed to explain the maximum in the blue end at high brightness levels, the latter to explain the maximum in the blue at low brightness levels. An examination of figs. 20 and 21 suggests, however, that as regards the maximum in the blue the curves are all members of a single family and can be generated from a single curve with the aid of one variable parameter, η_4. Thus we suggest there is a single common cause operative in different degrees

at high and low brightness levels, which produces in the Δ curve a maximum in the neighbourhood of $0 \cdot 46\ \mu$.

Expressed mathematically we assume that

$$\frac{(U_\lambda)\ \text{Fovea}}{(U_\lambda)\ \text{Parafovea}} = P(\eta_4, \lambda),$$



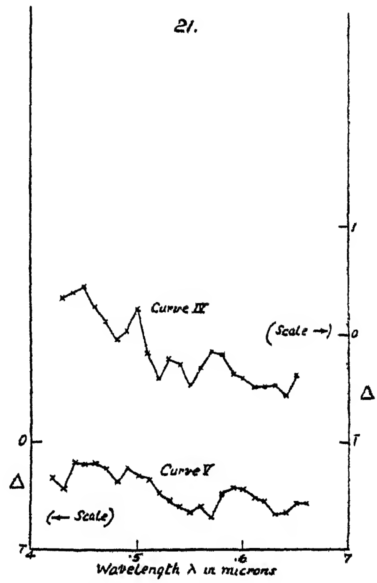


FIG. 20.—Logarithm of the ratio of the foveal to the 5°-parafoveal l.b.i. plotted against wave-length, *i.e.*, $\Delta = \log(1/U_\lambda)_{\text{parafoveal}} - \log(1/U_\lambda)_{\text{foveal}}$, against λ.

Curves I.—Data from figs. 6 and 7. Conditioning stimulation I. Δ, W. S. S.; ∇, B. H. C.

Curve II.—Data from fig. 16. Conditioning stimulation II, $B_s = 0 \cdot 3$ candles/sq. ft. white. ⊡, B. H. C.

Curves III.—Data from figs. 10 and 11. Conditioning stimulation II, $B_s = 300$ candles/sq. ft. white. O, W. S. S.; ×, B. H. C.

FIG. 21.—Δ against λ. Subject B. H. C.

Curve IV.—Data from fig. 15. Conditioning stimulation V.

Curve V.—Data from fig. 13. Conditioning stimulation III.

where η_4 is independent of the test spot wave-length and is dependent on the conditioning stimulation, and $P(\eta_4, \lambda)$ is a function with a peak at $0 \cdot 46$, the height of the peak depending on the value of η_4. Thus, for a parafoveal point,

$$1/U_\lambda = P(\eta_4, \lambda)\{\eta_1 X_1(\lambda) + \eta_2 X_2(\lambda) + \eta_3 X_3(\lambda)\},$$

and the state of a parafoveal region requires four adaptation potentials for its specification. There is a possibility that η_4 is a function of η_1, η_2 and η_3 in

which case three adaptation potentials suffice for the parafovea as for the fovea.

Physiologically $P(\eta_4, \lambda)$ may represent the effect of a sensitizing fluid, absent at the fovea, whose concentration is controlled by the conditioning stimulation.

The above discussion of the relation between parafoveal and foveal properties is, of course, very tentative and further work may show that the maximum in the $\triangle$ curve at about 0·46 μ may in fact originate from different causes at high and low brightness levels.

6. *Measurements for Coloured Conditioning Stimulations.*

The results of five single runs with different colour surrounds are shown in fig. 22. The brightness of the surround was adjusted in each run to equal approximately 25 candles/sq. ft. except for the blue-green surround (filter B′) for which by an error a brightness of 44 candles/sq. ft. was used.

Examining these curves we see that the curve for a white surround exhibits the characteristic three-peak form with which we are already familiar. In the second curve (blue surround, filter A′) the peak in the blue at about 0·44 μ has completely disappeared. In the third curve (red surround, filter F) there is no maximum or bulge suggestive of a maximum in the neighbourhood of 0·60 μ. Referring to equation (8) we may interpret the results in the following manner. With the white surround (η_1, η_2, η_3) assume a certain set of values. With the blue surround η_2 and η_3 are not altered very much, but η_1 is decreased to a much lower value. With the red surround, η_1 and η_2 are little changed, but η_3 is considerably reduced. Of the remaining curves (yellow surround, filter D; blue-green surround, filter B′), the yellow surround corresponds to practically the same relative values of η_1, η_2, η_3 as for white, The curve for a blue-green surround shows a relative lowering of η_1.

By removing the opaque stop u (fig. 2) a background brightness stimulation was obtained instead of a surround brightness stimulation. Two curves (single runs) for red and blue backgrounds respectively, are shown in fig. 23. The suppression of the blue and red peaks by blue and red backgrounds respectively, is evidenced in these curves.

Summing up the results obtained with a coloured conditioning stimulation, we may say that although only a few runs have been made, they do suggest that a relation similar to that of equation (8) may serve to represent the $\log(1/U_\lambda)$ curve for any conditioning stimulation. It will be the object of further work to follow up this suggestion and in particular to experiment with

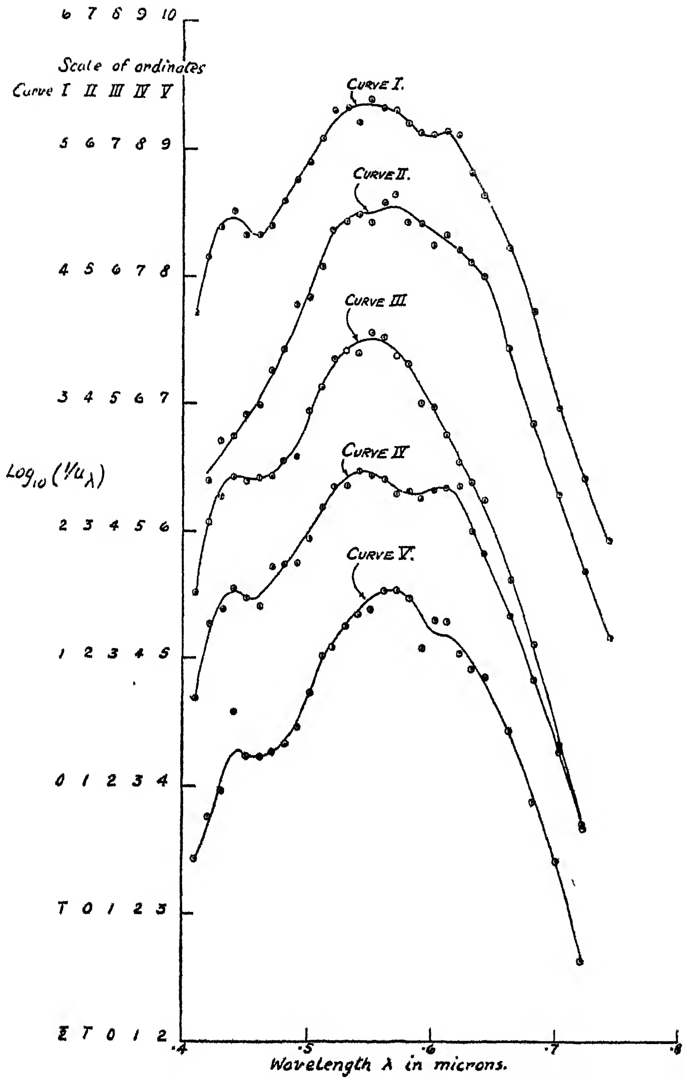


FIG. 22.—Log (I/U_λ) against λ for surround brightnesses of different colours. Single runs, B. H. C. fovea. Conditioning stimulation II. N = 6° (artificial pupil 3 mm. diameter).

Curve I.	B_s = 25 candles/sq. ft. white.
Curve II.	B_s = 26 candles/sq. ft. blue (filter A′).
Curve III.	B_s = 28 candles/sq. ft. red (filter F).
Curve IV.	B_s = 25 candles/sq. ft. yellow (filter D).
Curve V.	B_s = 44 candles/sq. ft. blue-green (filter B′).

approximately monochromatic surround and background brightnesses in place of the rather wide spectral bands which can be obtained with colour filters.

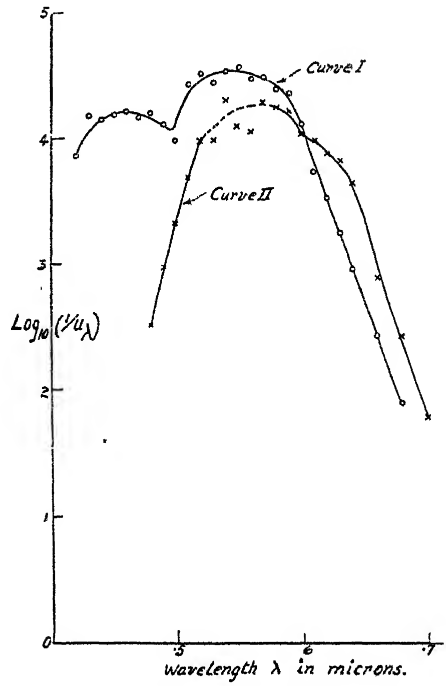


FIG. 23.—Log ($1/U_\lambda$) against λ for background brightnesses of different colours. Single runs, B. H. C. fovea. Conditioning stimulation V. N = 6° (artificial pupil 3 mm. diameter).

Curve I. $B = B_s$ = 34 candles/sq. ft. red (filter F).

Curve II. $B = B_s$ = 128 candles/sq. ft. blue (filter A′).

The authors have pleasure in thanking Sir John Parsons for his kindly interest in this work. The investigation was carried out under the auspices of the Illumination Research Committee of the Department of Scientific and Industrial Research.

LIST OF SYMBOLS.

Specification of Conditioning Stimulation.

B = brightness of 1·28° diameter centre field, expressed in candles/sq. ft.

B_s = brightness of an extended area surrounding the centre field, of outer diameter N = 30° or 6°. B_s in candles/sq. ft.

E = illumination in ft. candles produced on a vertical plane at the subject's eye, by a small bright source of light ($0 \cdot 8°$ diameter) placed $3°$ above the centre of the field.

r = distance from the artificial pupil to the background screen = 56 cm.

δ = area of the artificial pupil = $3 \cdot 14$ or $7 \cdot 03$ sq. mm.

A' = area of the centre field = $1 \cdot 22$ sq. cm.

Specification of the test stimulus and definition of the Liminal Brightness Increment.

u = intensity (brightness) of a test spot stimulus which is added to the centre field brightness, B.

$\{e_\lambda\}$ = relative energy distribution in test spot stimulus.

U = the critical value of u when the test spot is just visible to the subject. Thus U is the liminal brightness increment.

U_λ = U for the special case when the test stimulus is monochromatic and of wave-length λ. U_λ is expressed in absolute energy units, *i.e.*, in ergs/sec. per unit angular area of the test spot. U_λ is the liminal brightness increment for monochromatic light.

$\Delta\lambda$ = overall width of narrow spectral band serving as monochromatic light for the test spot.

A = area of the test spot = $0 \cdot 107$ sq. cm.

a = angular area of the test spot in degree units = $0 \cdot 70 \times 0 \cdot 16 = 0 \cdot 112$.

Δ = $\log (1/U_\lambda)_{\text{parafovea}} - \log (1/U_\lambda)_{\text{fovea}}$.

Symbols concerned with the Experimental Technique.

t_λ = transmission factor of the spectrometer for light of wave-length λ.

p_λ = dispersion of the spectrometer for light of wave-length λ.

b_λ and r_λ = transmission factors of stray light glasses used respectively in the blue and red ends of the spectrum.

v = voltage applied to the 100-watt projection lamp.

T_v = colour temperature of projection lamp when applied voltage is v.

$\{E_\lambda(v)\}$ = relative energy distribution in the radiation from the 100-watt projection lamp when applied voltage is v.

$I_{\lambda v}$ = flux of energy in ergs/sec. falling on the subject's eye when applied voltage is v.

$$D_{\lambda v} = \frac{C_2}{\lambda}\left\{\frac{1}{T_v} - \frac{1}{T_{100}}\right\}.$$

v_t = critical value of v when test spot is just visible. Thus $U_\lambda = I_\lambda(v_t)/a$.
C_2 = the exponential constant in Wien's law for the energy distribution of a black body radiator.
M = the mechanical equivalent of light = 15000 ergs/lumen sec.
V_λ = relative luminosity function for the light-adapted eye (values adopted by the International Commission on Illumination, Paris, 1921).

Symbols introduced in the Discussion of the Results.

η_1, η_2, η_3 = three variable parameters, independent of wave-length but dependent on the conditioning stimulation, which enter in the general expression (8) giving $1/U_\lambda$ for the foveal retina under all conditions.
$X_1(\lambda), X_2(\lambda), X_3(\lambda)$ = three fixed functions of wave-length, which enter in the expression (8).
η_4 = a fourth variable parameter occurring in the expression (11) giving $1/U_\lambda$ for the parafoveal retina.
$P(\eta_4, \lambda)$ = antilog Δ = the ratio of the parafoveal to the foveal value of $1/U_\lambda$ for a given conditioning stimulation.
σ = numerical result of any localized visual test.*
μ = symbol representing all the test variables which play a part in determining σ.*
$\xi_1, \xi_2 \ldots, \xi_n$ = the adaptation potentials, whose values at a given point of the retina represent the conditioning stimulation in the formula for all localized visual tests.*
β = the equivalent background brightness of a prescribed conditioning stimulation, or the uniform field brightness which has the same effect on the numerical result of any test, as the given conditioning stimulation (white light).*

Summary.

The eye was brought to a definite condition by viewing a known distribution of brightness in the visual field, made up of a small centre field of uniform brightness B surrounded by an area of uniform brightness B_s, together with, in some cases, a bright spot of light 3° above the centre of the field. Concentric with the centre field, a small rectangular patch (0·7° × 0·16°) of monochromatic

* See Stiles and Crawford (1932).

light of variable brightness was presented to the subject's view and the measurements consisted in determining the least brightness (U_λ) of this patch such that the subject could just detect its presence. The minimum brightness U_λ has been termed the "liminal brightness increment" and in this work its value has been determined in absolute energy units for various wave-lengths λ throughout the spectrum. The value of U_λ obtained depends on whether the subject views the test patch by direct (foveal) vision or whether he directs his gaze away from the centre of the field and perceives the test patch by parafoveal or peripheral vision. The cases studied here are foveal and 5°-parafoveal vision. The results are expressed by plotting $\log (1/U_\lambda)$ against λ for each condition of the eye studied and for foveal and 5°-parafoveal vision. The foveal and parafoveal curves of $\log (1/U_\lambda)$ against λ obtained with the totally dark-adapted eye ($B = B_s = 0$), have the same shape as curves obtained under similar conditions by Abney and Watson; for wave-lengths greater than $0 \cdot 62\ \mu$ foveal and parafoveal values are nearly the same, but as shorter wave-lengths are approached the parafoveal value of $\log (1/U_\lambda)$ becomes increasingly greater than the foveal value, *i.e.*, the parafovea becomes much more sensitive than the fovea. For the eye adapted to high brightness levels of white light (*e.g.*, $B_s = 300$ candles/sq. ft.), the curve of $\log (1/U_\lambda)$ against λ exhibits a characteristic form with maxima at or near $\lambda = 0 \cdot 44\ \mu$ and $\lambda = 0 \cdot 54\ \mu$ and with a third maximum or bulge in the neighbourhood of $\lambda = 0 \cdot 60\ \mu$. Parafovea and fovea behave similarly in this respect although the parafovea still has a somewhat greater senstitivity in the blue end of the spectrum. With the eye adapted to high brightnesses of coloured light the $\log (1/U_\lambda)$ against λ curve is modified by the suppression or partial suppression of one of the three maxima. For example, with $B_s = 25$ candles/sq. ft. of blue light the maximum at $0 \cdot 44\ \mu$ is absent, the shape of the curve for wave-lengths greater than $0 \cdot 50\ \mu$ being nearly the same as for $B_s = 25$ candles/sq. ft. of white light. A close connection of the three maxima at $0 \cdot 44\ \mu$, $0 \cdot 54\ \mu$ and $0 \cdot 60\ \mu$, with the three mechanisms postulated by the trichromatic theory, is indicated.

Appendix.

Determination of the Transmission of the Spectrometer at Different Wave-lengths.

The transmission of the spectrometer (Spectrometer I) used in this work was determined with the help of a second spectrometer. The arrangement of the necessary optical parts is shown diagrammatically in fig. 24. The slits of the instrument under test were removed after the preliminary adjustment

had been carried out. Lamp A was run at a constant voltage throughout the determination. The voltage on the comparison lamp J was varied to give a brightness match in the image of the photometric field L seen by the eye. The lines of division of the photometric field had to be arranged at right angles to the spectrometer slits; in all other positions they became broadened and fuzzy owing to diffraction at the slits. Lamp J had previously been calibrated

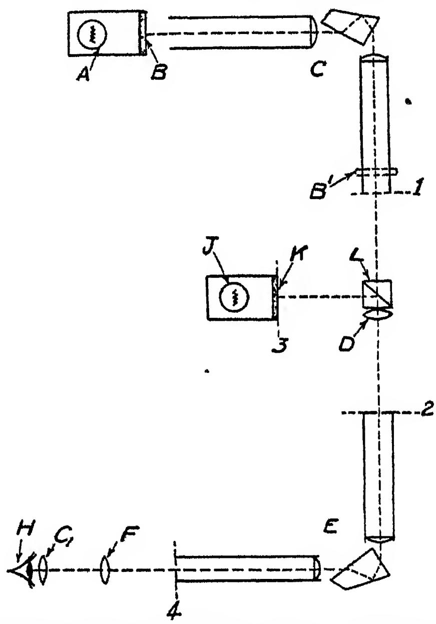


Fig. 24.—Plan of a paratus for the measurement of the transmission of the spectrometer. A, lamp (100-watt projector type). B, opal glass. C, spectrometer under test. D, lens focussing planes 1 and 3 at plane 2. E, auxiliary spectrometer for isolating any desired wave-length. F, lens focussing plane 4 at the observer's eye. C_1, eye lens to enable observer to focus image of photometric field. H, observer's eye. J, comparison lamp (calibrated for voltage-intensity relationship). K, opal glass. L, Lummer-Brodhun cube. B′, second position of opal B.

against standard sector discs so that the relation between voltage and intensity at any wave-length was known. A series of measurements was first made at a number of wave-lengths throughout the spectrum with the apparatus set up as in the diagram. Spectrometer C was then removed and opal B with its lamp brought to position B′. A second series of measurements at the same wave-lengths was then made. From the two series the transmission of the spectrometer C could be calculated for each wave-length used. In the following table are given the measured transmissions for a Hilger wave-length spectrometer (Spectrometer I) and a Hilger-Tutton monochromatic illuminator (Spectrometer II), both instruments being of the constant deviation type.

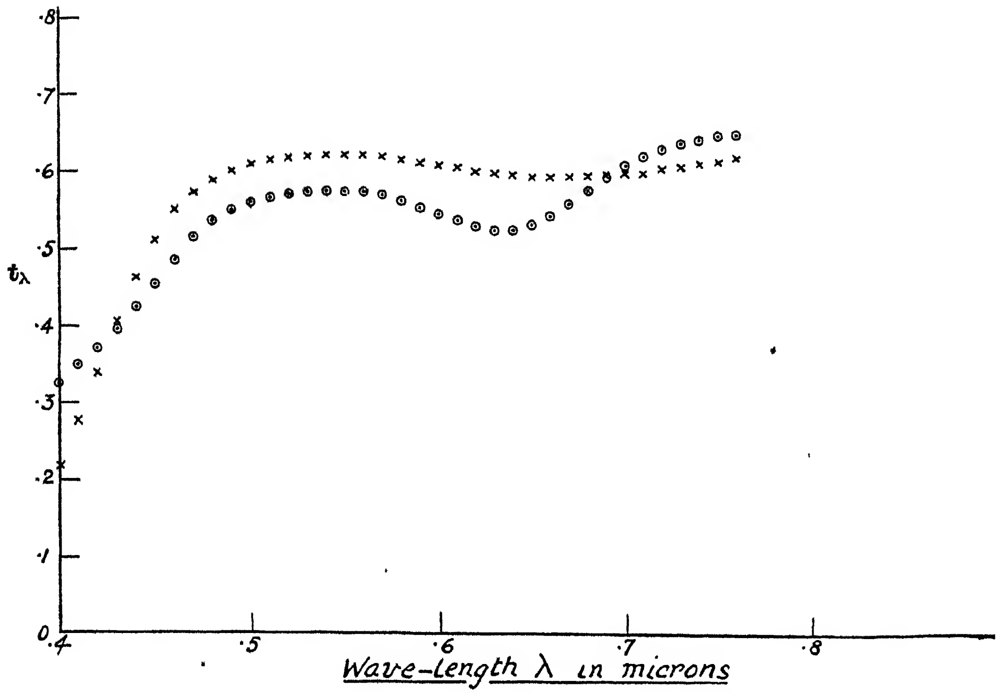


FIG. 25.—Tranmisssion factor t_λ as measured for two spectrometers. Spectrometer I was used in the main apparatus. ×, Hilger wave-length spectrometer (Spectrometer I); ⊙, Hilger-Tutton monochromatic illuminator (Spectrometer II).

	Transmission.			Transmission.	
λ.	Spectrometer I.	Spectrometer II.	λ.	Spectrometer I.	Spectrometer II.
μ.			μ.		
0·40	0·217	0·325	0·59	0·617	0·557
0·41	0·275	0·348	0·60	0·612	0·548
0·42	0·338	0·369	0·61	0·610	0·540
0·43	0·404	0·395	0·62	0·605	0·532
0·44	0·461	0·423	0·63	0·603	0·527
0·45	0·511	0·454	0·64	0·600	0·528
0·46	0·550	0·486	0·65	0·598	0·536
0·47	0·574	0·515	0·66	0·598	0·547
0·48	0·590	0·537	0·67	0·600	0·564
0·49	0·603	0·551	0·68	0·601	0·581
0·50	0·611	0·561	0·69	0·603	0·600
0·51	0·617	0·568	0·70	0·604	0·615
0·52	0·619	0·573	0·71	0·605	0·627
0·53	0·622	0·575	0·72	0·610	0·638
0·54	0·624	0·577	0·73	0·614	0·646
0·55	0·624	0·577	0·74	0·618	0·650
0·56	0·624	0·577	0·75	0·622	0·655
0·57	0·622	0·573	0·76	0·627	0·658
0·58	0·619	0·566			

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Studies on the Hypophysectomized Ferret. IV.—*Comparison of the Reproductive Organs during Anœstrus and after Hypophysectomy.*

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[PLATE 13.]

I. *Introduction.*

In Part II (Hill and Parkes, 1932, *b*) it was shown that hypophysectomy of the male ferret during the breeding season caused the testes to regress to a condition very similar to that found in the normal animal during anœstrus. This result suggested that decreased activity of the anterior pituitary body might be the causative factor in anœstrus. The hypophysectomized ferrets regressed from full activity to the anœstrous condition in about one month, *i.e.*, much more rapidly than the normal animal regresses at the end of the

breeding season. This fact showed that hypophyseal activity diminishes gradually at the end of the breeding season and presumably reaches its lowest level when the testes are most atrophic in November.

The next problem was to determine whether or not hypophysectomy during November leads to further regression, *i.e.*, whether the pituitary body is wholly or only partially inactivated at the extreme of the anœstrous period. We have now investigated this question, and also carried out similar experiments on the female.

II. *Technique.*

Hypophysectomy was performed as described previously (Hill and Parkes, 1932, *a*). The completeness of the operation was determined by serial sections of the sellæ turcica. In describing the results, "complete" means that there is no reasonable doubt; "? fragment" means that a few cells were found which may have been of anterior pituitary origin but which were certainly not of functional significance. Histological technique was as usual. The gonads and accessory organs were weighed from 70% alcohol during upgrading. The diameter of the Graafian follicle is given as the mean of two diameters taken at right angles, while that of the testis tubule is the mean for 10 tubules measured at right angles to the longitudinal axis. The weight given for ovaries and testes refer to the total weight of the pair.

III. *The Male Ferret.*

Condition during Anœstrus.—The cyclic changes in the normal ferret testis have been described by Allanson (1932). Some additional data for size comparison is given here in Table I. During February–July inclusive the testes are fully developed and weigh about 3 gm., the tubules approaching 200 μ in diameter. The extreme point of anœstrus is reached in November, when the testes weigh less than 0·5 gm., and the tubule diameter is slightly less than 100 μ. At this time only Sertoli cells, spermatogonia and a few primary spermatocytes are present in the tubules.

Condition after Hypophysectomy.—Four ferrets were hypophysectomized in November and killed at periods up to 15 weeks later. The data relating to these animals are summarized in Table II. Comparison of these results with the control data given by Allanson (1932) and also in Table I shows that the removal of the hypophysis during anœstrus effectively prevents the recovery of the testes from the atrophic anœstrous condition. On the other hand, the

operation does not accentuate the size decrease normally found in anœstrus. This similarity of the testes of anœstrous and hypophysectomized anœstrous ferrets is also shown by the tubule diameter, which is comparable in the two types. Histologically, the testes of DHF1, 4 and 5, are indistinguishable from each other. The great majority of tubules contain only Sertoli cells and spermatogonia, which, in section, form an irregular ring inside the basement membrane. The centre of the tubules is occluded by the Sertoli syncytium, figs. 1 and 2, Plate 13. Few or no tubules contain primary spermatocytes, and the testes after hypophysectomy may thus be slightly more atrophic than during ancestrus. The typical "winter-gonia" described by Allanson are present. The testes of these three animals at this stage differ from that of ferret MHF2 (see Part II) at 30 days after hypophysectomy in having

Table I.—Control Male Ferrets.

Number of animal.	Date killed.	Testes weight, gm.	Epididymides weight, gm.
DHF 2	November 2	0·363	0·117
3	November 2	0·395	0·155
6	November 6	0·519	0·163
THF 3	November 9	0·429	0·164
5	November 9	0·433	0·147
9	November 19	0·282	0·081
14	February 16	2·02	0·250
15	February 22	2·45	0·380
16	February 23	2·12	0·290
17	February 23	2·89	0·310
18	February 25	2·01	0·334
19	February 25	3·35	0·422

Table II.—Hypophysectomized Male Ferrets.

No. of animal.	Date of hypophysectomy.	Time of killing after hypophysectomy, days.	Testes.			Epididymides weight, gm.	Completeness of hypophysectomy.
			Weight, gm.	Diameter tubules, μ.	Condition.		
DHF 7	Nov. 7	78	0·554	103	Slightly active	0·205	Fragment.
4	Nov. 3	99	0·313	91	Atrophic	0·212	? fragment.
5	Nov. 5	101	0·319	97	,,	0·222	Complete.
1	Nov. 1	105	0·390	98	,,	0·262	,,

fewer tubules containing spermatocytes. This is almost certainly due to the greater time after hypophysectomy.

The testes of DHF 7 are less atrophic, many tubules containing primary and secondary spermatocytes and some a definite lumen. This more developed condition is presumably due to the fragment of anterior lobe tissue found in the sella at autopsy.

The epididymides of all four animals were similar to those of the normal anœstrous male in that they showed—(*a*) small diameter of the tubule; (*b*) low epithelium with degenerate cilia; (*c*) a relatively large proportion of fibrous and muscular tissue, and (*d*) complete absence of spermatozoa.

The fact that removal of the pituitary body during anœstrus does not appreciably increase the atrophy of the testis suggests most strongly that the pituitary body of the normal ferret is practically inactive during the short period in November, when anœstrous atrophy of the testis reaches its extreme point.

If this material dealing with the size of the testis in the third and fourth months after hypophysectomy is combined with that showing the changes in the first month (Hill and Parkes, 1932) it is possible to obtain some idea of the course of post-hypophysectomy atrophy. The two sets of animals are not really serial because of the different initial condition of the testes in June and in November, but comparing Table II above and Table I in Part II (Hill and Parkes, 1932, *b*) it may be concluded that (*a*) whereas the minimum size of testis tubule is attained within one month of hypophysectomy of the fully functional animal, the minimum gross size of the testis is not reached so soon; and that (*b*) changes in the germ cells are negligible after the first month and relate only to decrease in the number of spermatocytes.

IV. *The Female Ferret.*

Condition during Anœstrus and Œstrus.—The normal reproductive cycle in the female ferret has been dealt with by Hammond and Marshall (1930). These authors found that the diameter of the largest follicles in anœstrus varies greatly according to the time of year, being, in the most retrogressive stage, only about 0·24 mm. At this size the follicle is still usually solid in the ferret (Parkes, 1931). Our normal anœstrous material is summarized in Table III.* In October, November and December, the ovaries weigh about 50 mg. Variation, however, is considerable, and is partly due to accumulation of interstitial tissue in the second year and older animals. Many of the*

anœstrous ovaries contain small masses of cells which probably originate from the atresia of small follicles, fig. 5, Plate 13.

In January, the ovaries of some of the more forward animals are beginning to show histological signs of activity, but not until February and March is there any general increase in follicular size. At œstrus, the weight of the ovaries has increased to 100–200 mg., and the size of the ripe follicle is 1·2–1·4 mm.

Table III.—Control Female Ferrets.

No. of ferret.	Date killed.	Parous or non-parous.	Ovary.		Uterus.	
			Weight, gm.	Size of largest follicle, mm.	Weight, gm.	Condition.
GHF 11	October 28	P.	0·048	0·25	—	0
DHF 12	November 15	N.-p.	0·043	—	0·101	0
EHF 5	November 24	P.	0·055	n.a.	—	0
6	November 30	N.-p.	0·065	0·27	0·107	0
CF 4	December 6	N.-p.	0·024	0·29	0·067	0
5	December 6	P.	0·039	0·31	0·174	0
6	December 6	N.-p.	0·053	0·34	0·077	0
EHF	January 6	P.	0·058	0·26	0·186	0
24	January 11	N.-p.	0·075	0·51	0·170	+
25	January 11	N.-p.	0·144	0·65	0·152	+
29	January 12	P.	0·047	0·39	0·095	0
30	January 19	P.	0·043	0·43	0·132	0
34	January 25	N.-p.	0·035	0·42	0·097	0

P = Parous. N.-p. = Non-parous. n.a. = none with antrum.

During anœstrus the uterus weighs up to 200 mg., considerable variation being found according to the previous history. The normal non-parous anœstrous uterus, fig. 3, Plate 13, has poorly developed serous and muscular coats, while the endometrium is thin and almost devoid of glands; such uteri often weigh only 50 mg. The parous anœstrous uterus is much larger, and the serous covering contains fibrosed blood vessels diagnostic of a previous pregnancy. The endometrium is dense and shows the remains of old glandular development. Both types of anœstrous uteri are poorly vascularized and are designated as condition 0 in the tables in this and the following papers. The first breeding season change in the uterus is that the stroma next to the muscle layer becomes œdematous and superficial glands begin to develop from the lumen, condition +. Subsequently, the whole endometrium increases in size, the glands grow down into the œdematous stroma and the uterus becomes plump in appearance, condition ++. At this stage the glands form a thick

layer surrounding the lumen with œdematous stroma beneath. Later in this process, practically all the endometrium is occupied by glands and the stroma becomes much less obvious, condition +++. The glands, however, are still compact. Finally the basal ends of the glands enlarge and the whole endometrium acquires a broken-up appearance as the gland ducts expand, condition ++++. At this stage the uterus weighs up to about 1·0 gm. When the uterus is between stage ++ and +++ the vulva begins to swell, and most animals are in full œstrus early in April.

Condition after Hypophysectomy.—Four female ferrets, hypophysectomized in anœstrus and kept for periods up to 90 days afterwards, are available for comparison with the normal ones described above. The data relating to these four animals are summarized in Table IV. The ovaries are all abnormally small; the largest being about the same size as the smallest of those of the normal anœstrous animals. This small size is a reflection of the almost total absence of follicles with antra. The uteri were all small except that of DHF 8, a parous animal.

Table IV.—Hypophysectomized Female Ferrets.

No. of animal.	Date of hypophysectomy.	Time of killing after hypophysectomy, days.	Parous or non-parous.	Ovary.		Uterus.		Completeness of hypophysectomy.
				Weight, gm.	Size of largest follicle, mm.	Weight, gm.	Condition.	
DHF 8	November 8	57	P.	0·022	n.a.	0·186	0	Complete.
11	November 11	55	N.-p.	0·028	n.a.	0·085	0	"
13	November 16	90	N.-p.	0·015	0·250	0·066	+	Fragment.
16	November 26	76	N.-p.	0·027	n.a.	0·070	0	Complete.

P = Parous. N.-p. = Non-parous. n.a. = none with antrum.

Histologically, the ovaries of DHF 8, 11 and 16 were degenerate, fig. 6, Plate 13, the least so being more atrophic than the normal anœstrous ovary. These three animals all possessed large numbers of young ova and also fully grown ova surrounded by a few layers of follicular epithelium, but no later stages of follicular growth were seen. From these results it seems probable that the very early growth of the ovum and follicle is independent of the anterior pituitary body. The uteri of these animals were in keeping with the ovaries, all being atrophic, fig. 4, Plate 13.

The ovaries of DHF 13, however, showed slight follicular development, largest follicle 250 μ, and the uterus also appeared active. These findings are

consistent with the presence of a small fragment of possibly secretory tissue found in the sella.

These females were killed in February, when appreciable development of follicles should have been taking place, and it is evident that removal of the pituitiary body completely inhibits the development of the breeding season changes in the female ferret.

The ovary is slightly more degenerate 2–3 months after hypophysectomy than during anœstrus. The difference, however, may well be due to the longer duration of the operative than of the natural quiescence, and it is so slight that the anterior pituitary body, as in the male ferret, would appear to be practically inactive during the short time when the extreme anœstrous retrogression is found.

IV. *Summary.*

(1) Hypophysectomy of the ferret during anœstrus inhibits the subsequent onset of breeding season changes.

(2) The atrophic condition of the reproductive organs of both males and females during anœstrus is only slightly accentuated by the removal of the hypophysis at this time.

(3) It seems probable, therefore, that the pituitary body of the ferret is practically inactive during the extreme of anœstrus.

DESCRIPTION OF PLATE.

Fig. 1.—Testis of DHF 5, hypophysectomized anœstrous, showing atrophic condition and absence of primary spermatocytes. × 66.

Fig. 2.—Testis of DHF 1, hypophysectomized anœstrous, showing contents of tubule. × 160.

Fig. 3.—Uterus of EHF 34, normal anœstrous. × 17.

Fig. 4.—Uterus of DHF 16, hypophysectomized anœstrous, showing condition similar to EHF 24. × 17.

Fig. 5.—Ovary of GHF 11, normal anœstrous, showing almost complete absence of follicles with antra. × 17.

Fig. 6.—Ovary of DHF 11, hypophysectomized anœstrous, showing presence of oocytes in all stages of growth, but absence of follicles with antra. × 17.

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612.826.3 : 612.6.

Studies on the Hypophysectomized Ferret. V.—Effect of Hypophysectomy on the Response of the Female Ferret to Additional Illumination during Anœstrus.

By MARGARET HILL (Keddey Fletcher-Warr Student) and A. S. PARKES, F.R.S. (Foulerton Student of the Royal Society).

(From the National Institute for Medical Research, London, N.W.3.)

(Received June 28, 1933).

[PLATE 14.]

I. *Introduction.*

Bissonnette (1932), following up his work on the European starling, recently reported that anœstrous ferrets could be brought into œstrus by exposure to additional illumination. The animals were given 6–6½ hours electric light each evening from October 12 onwards. Full œstrus occurred in 38–64 days, *i.e.*, well within the normal anœstrous period, and ovulation followed copulation. Males subjected to similar treatment showed endocrine activity of the testis, with resulting willingness to copulate, but spermatogenesis failed to progress beyond the secondary spermatocyte stage.

In view of the general correlation between the gonads and the anterior pituitary body and of the activation of the anœstrous female ferret by injection of anterior lobe preparations (Hill and Parkes, 1930), it seemed probable that the effects reported by Bissonnette were due to stimulation of the anterior lobe by the light treatment. If this were so the response to light should be obliterated by hypophysectomy. Experiments carried out on these lines on female ferrets are reported below. In the course of the work, we have fully confirmed Bissonnette's observations on the induction of œstrus in the anœstrous ferret by additional illumination.

II. *Technique.*

Operative, histological and other techniques were as usual. The degrees of activity of the uterus are classified as described in IV, p. 530.

Illumination.—The conditions of illumination were made to correspond as closely as possible to those employed by Bissonnette. Two blocks of six cages were each illuminated by two 100-watt electric light bulbs placed above one another on a standard about 2½ feet from the front of the cages. The lights

were so arranged that no part of the cage was in shadow. No bedding other than sawdust was allowed. The lights were turned on from 4.30 p.m. till 11.0 p.m. The duration of the light treatment is given in Tables I and II.

III. *Effect of Light on Normal and Hypophysectomized Anœstrous Female Ferrets.*

Five normal female ferrets were given additional light from October 31 onwards. One was taken off after 29 days, and two more were put on. The results are shown in Table I.

Table I.—Normal Ferrets receiving Evening Illumination during November and December.

No. of animal.	Parous or non-parous.	Illumination begun.	Killed, days after illumination begun.	Ovaries.		Uterus.		Vulva.
				Weight, gm.	Size largest follicle, mm.	Weight, gm.	Condition.	
GHF 14	N.-p.	Oct. 31	65	0·087	1·3	0·672	++++	Up.
15	N.-p.	,,	65	0·053	1·3	0·690	++++	Up.
16	P.	,,	29	0·119	0·45	0·288	+	No sign.
17	N.-p.	,,	65	0·060	1·35	0·685	++++	3/4 up.
18	P.	,,	65	0·115	0·63	0·663	++	No swelling.
19	P.	Nov. 29	51	0·070	1·3	0·733	++++	Up.
20	P.	,,	51	0·104	1·6	0·822	++++	3/4 up.

P = Parous. N.-p. = Non-parous.

The last two animals were killed on January 19, at which time the first signs of pre-breeding season development have scarcely appeared in normal animals (see Table III, Part IV). Five of the seven illuminated ferrets showed complete œstrous development of the ovaries and complete or practically complete hypertrophy of the uterus and vulva. Of the remaining two, GHF 16, illuminated for only 29 days, showed slight follicular and appreciable uterine development, while GHF 18, showed appreciable follicular and uterine development, but no growth of the vulva.

The results obtained in the six operated animals, illuminated under identical conditions, are given in Table II. Two of these ferrets responded to the treatment, and the ovaries, uterus and vagina developed the typical symptoms of œstrus. These two were found to be incompletely hypophysectomized, appreciable pieces of anterior lobe tissue being found on sectioning the sellæ. A third animal, GHF 7, showed some development of the ovaries and uterus ;

Table II.—Hypophysectomized Ferrets receiving Evening Illumination during November and December.

No. of animal.	Parous or non-parous.	Date of hypophysectomy.	Illumination begun.	Killed, days after illumination begun.	Ovaries.		Uterus.		Vulva.	Completeness of hypophysectomy.
					Weight, gm.	Size largest follicle, mm.	Weight, gm.	Condition.		
GHF 5	P.	October 19	October 31	58	0·038	n.a.	0·206	0	No sign	? Minute fragment.
7	P.	October 24	October 31	65	0·050	0·56	0·265	+	,,	Probably fragment.
8	N.-p.	October 25	October 31	65	0·021	n.a.	0·061	0	,,	Complete.
9	N.-p.	October 25	October 31	65	0·052	1·42	0·732	++++	Up	Definite piece present.
10	N.-p.	October 26	October 31	65	0·051	1·18	0·660	++++	,,	,, ,,
12		October 28	October 31	65	0·015	n.a.	0·055	0	No sign	Complete.

P = Parous. N.-p. = Non-parous.

sections of the sella revealed what was probably a fragment of anterior lobe. The remaining three animals showed no response whatever to the light treatment, and maintained the typical inactive condition described in IV. Of these three, two were completely hypophysectomized, while the third had a minute fragment of tissue, possibly, but not certainly, identifiable as anterior lobe.

These results would appear to show quite definitely that the response of the anœstrous female ferret to additional illumination is dependent upon the presence of anterior pituitary tissue. It is reasonable to assume, therefore, that the response to light treatment is caused by activation of the anterior pituitary body, which in turn activates the reproductive organs. It is difficult to understand by what route additional illumination acts upon the pituitary body, but an obvious experiment would be to investigate the reaction of blind ferrets.

IV. *Summary.*

Exposure to additional illumination induces œstrus in the anœstrous female ferret (Bissonnette, 1932). This reaction is inhibited by hypophysectomy and is probably due to stimulation of the anterior pituitary body by the light treatment.

DESCRIPTION OF PLATE.

FIG. 1.—Uterus of GHF 19, illuminated anœstrous, showing beginning of final stage of glandular development typical of œstrus. × 20.

FIG. 2.—Ovary of GHF 19, illuminated anœstrous, showing ripe follicles. × 20.

FIG. 3.—Uterus of GHF 8, hypophysectomized illuminated anœstrous, showing absence of development. × 20.

FIG. 4.—Ovary of GHF 8, hypophysectomized illuminated anœstrous, showing absence of follicular development. × 20.

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612.826.3:612.6

Studies on the Hypophysectomized Ferret. VI.—*Comparison of the Response to Œstrin of Anœstrous, Ovariectomized and Hypophysectomized Ferrets.*

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(From the National Institute for Medical Research, London, N.W.3.)

(Received June 28, 1933).

[Plate 15.]

I. *Introduction.*

Most of the work dealing with the relation between the anterior pituitary body and the reproductive organs has tended to show that changes in the accessory reproductive organs in hyper- or hypo-pituitarism are merely secondary to changes in the gonads. If this is so, the atrophy of the vagina and uterus after hypophysectomy should be no greater than after ovariectomy, and the sensitivity to œstrin in the two conditions should be similar. On the other hand, certain work has suggested that the substances secreted by the anterior lobe may have some direct effect on the accessory organs, so that the uterus and vagina of the hypophysectomized animal might be less sensitive to œstrin than those of the ovariectomized animal, especially if the administration of œstrin has any stimulating action on the anterior lobe. Accordingly, Smith (1932) investigated the comparative sensitivity of the two types in the rat, and was unable to demonstrate any essential difference.

We have been led to consider the same problem in hypophysectomized ferrets from the point of view of sensitizing the uterus to the action of the corpus luteum hormone. We have injected with œstrin three types of ferret, anœstrous, ovariectomized anœstrous and hypophysectomized anœstrous, in all of which the appearance of the accessory organs is similar (IV, p. 530).

II. *Technique.*

Operative and Histological Technique.—Hypophysectomies and ovariectomies were performed as before. Histological examination of the uteri was carried out by cutting each cornu into three or more pieces and embedding so that six sections were obtained from different parts of the uterus at the same time.

Some such procedure is necessary in view of the variation found in different parts of the uterus. The states of development of the uteri have been classified as described in IV, p. 530.

Œstrin.—Crystalline trihydroxy-œstrin was used for all experiments. This substance has an activity of about 8000 mouse units per milligram on the Marrian-Parkes (1929) method of assay. An aqueous solution was used for most of the injections; this was made by taking up the crystalline hormone in absolute alcohol, and throwing the alcoholic solution into distilled water. By this means very dilute solutions quite suitable for injection could be obtained having 100 gamma or about 800 mouse units per c.c. All injections were subcutaneous.

III. *Experimental Results.*

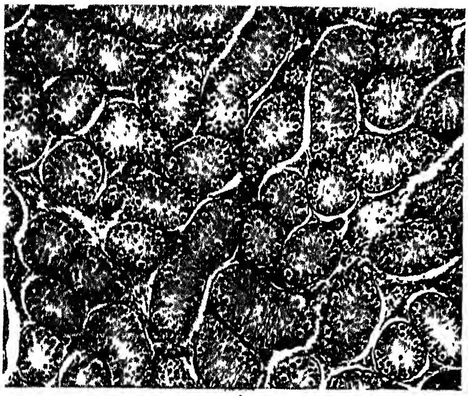
The uterus of the ferret is known to show appreciable development in response to amounts of œstrin which have no effect upon the vulva (Parkes, Rowlands and Brambell, 1932), and for comparative purposes it seemed desirable to give sufficient œstrin to cause only slight swelling of the vulva. To this end, a small daily dose was first given, this being doubled at later injections. The full details of the experiments are given in Tables I, II, III.

Table I.—Effect of Œstrin on Anœstrous Ferrets.

No. of ferret.	Date injection begun.	Days œstrin given.	Total amount given, mg.	Weight ovaries, mg.	Uterus.		Vulva.
					Weight, gm.	Condition.	
EHF 26	Jan. 19	12	1·4	0·050	0·495	+++	Slightly up.
27	,, ...	16	2·2	0·044	0·755	++	1/3 up.
28	,, ...	16	2·2	0·030	0·545	+++	1/4 up.

Table II.—Effect of Œstrin on Ovariectomized Anœstrous Ferrets.

No. of ferret.	Date of ovariectomy and first injection.	Days œstrin given.	Total amount given, mg.	Uterus.		Vulva.
				Weight, gm.	Condition.	
EHG 31	January 19	16	2·2	0·450	++	1/4 up.
32	,, ...	16	2·2	0·498	++	1/4 up.
33	,, ...	16	2·2	0·407	++	1/3 up.



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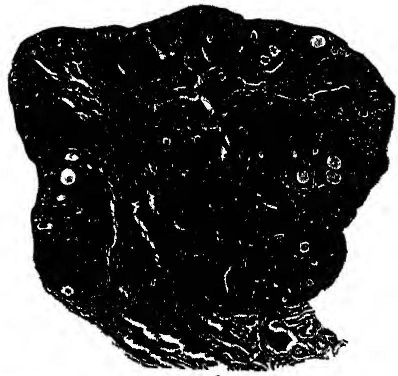

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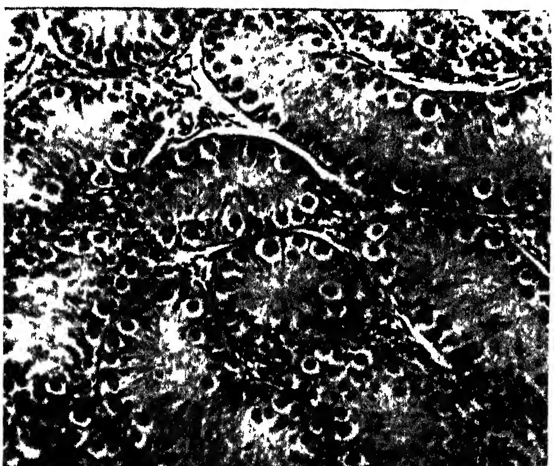

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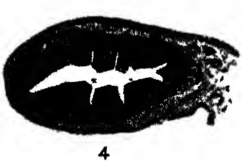

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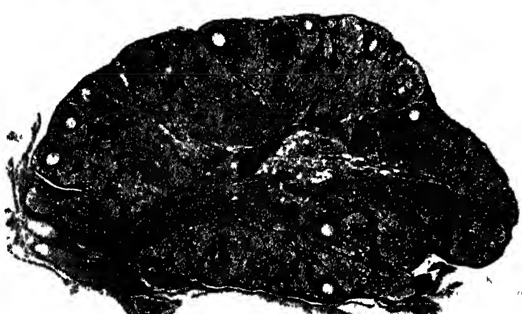

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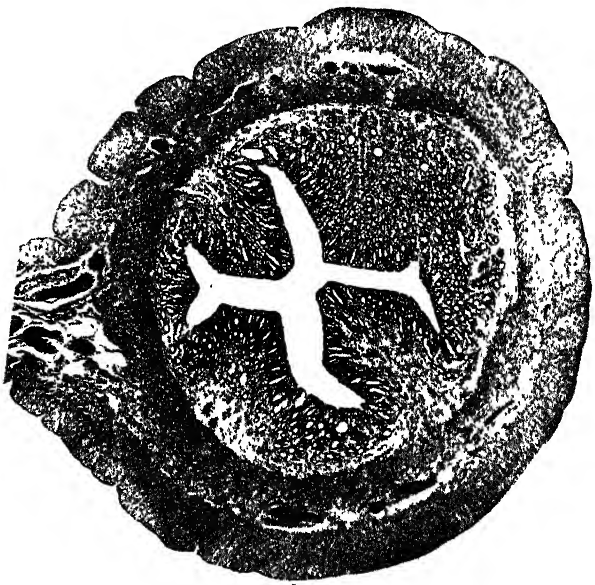

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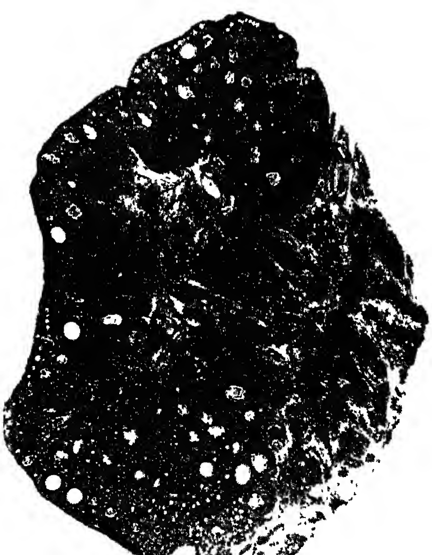

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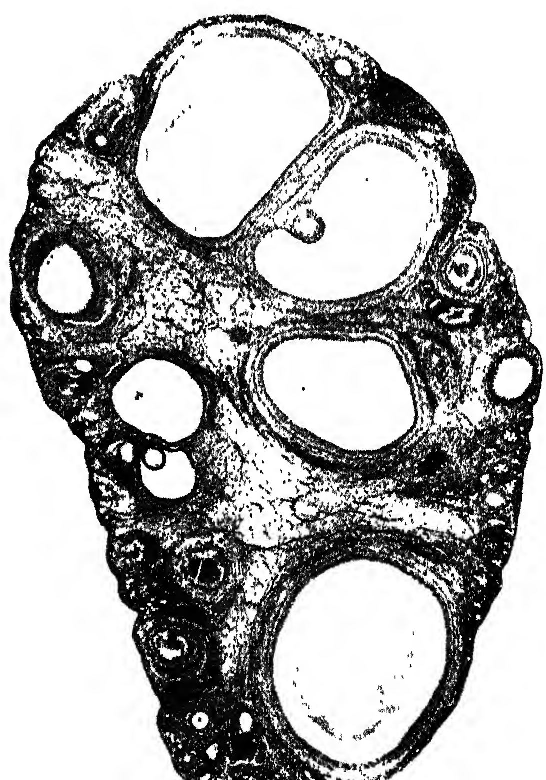

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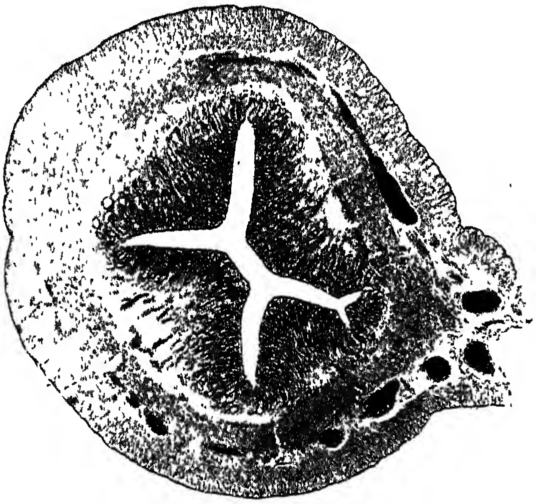

FIG. 1.

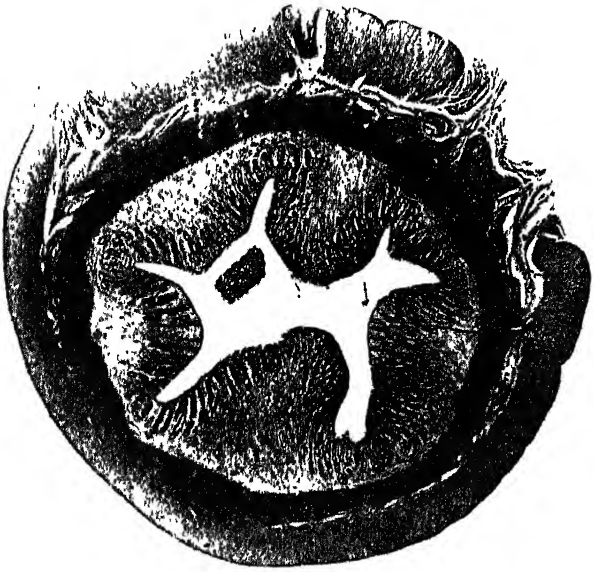

FIG. 2.

FIG. 3.

Table III.—Effect of Œstrin on the Hypophysectomized Anœstrous Ferret.

No. of ferret.	Date of hypophysectomy.	Time injection begun, days after hypophysectomy.	Days œstrin injected.	Total amount given, mg.	Weight ovaries, gm.	Uterus.		Vulva.	Completeness of hypophysectomy.
						Weight, gm.	Condition.		
EHF 16	Dec. 14	36	5	0·5	0·035	0·627	+	No sign	Complete
20	Dec 22	28	12	1·4	0·026	0·430	+++	Slight swelling	„
15	Dec. 13	37	16	2·2	0·028	0·435	++	1/3 up	„
21	Dec. 30	25	15	2·0	0·023	0·297	++	1/4 up	„

To judge by any one of the obvious criteria, (*a*) weight of uterus, (*b*) development of endometrium, or (*c*) growth of the vulva, the responses of the three types of animal to œstrin are essentially similar. There is certainly no fundamental difference in the sensitivity of the uteri of the hypophysectomized animals. The full œstrous condition was not produced in any of the uteri, and we have yet to ascertain whether the degree of morphological development attained represents adequate sensitivity to the action of the corpus luteum hormone.

Some earlier results with castor oil solution of œstrin may be recorded. The animals used were both ovariectomized and hypophysectomized, but in view of the experiments recorded above, the dual operation can have added little to the insensitivity of the uterus. Two such ferrets received 10·1 mg. of œstrin in castor oil over 18 days and failed to show any development of vulva or uterus. The third received 13·9 mg. over 32 days and showed only slight development of both. This experiment, compared with those in which an aqueous solution was used, would appear to be a further demonstration of the inefficiency of oil as a medium for the injection of œstrin.

IV. *Summary.*

(1) Three types of female ferrets were injected with trihydroxy-œstrin, (*a*) normal anœstrous, (*b*) ovariectomized anœstrous, and (*c*) hypophysectomized anœstrous.

(2) There appears to be no essential difference between the three types in the sensitivity of the uterine endometrium to the hormone.

DESCRIPTION OF PLATE.

Fig. 1.—Uterus of EHF 26, normal anœstrous, receiving 1·4 mg. œstrin in 12 days. × 21.

Fig. 2.—Uterus of EHF 32, ovariectomized anœstrous, receiving 2·2 mg. œstrin in 16 days. × 21.

Fig. 3.—Uterus of EHF 21, hypophysectomized anœstrous, receiving 2·0 mg. œstrin in 15 days. This uterus is the smallest of the hypophysectomized series, but the degree of glandular development is similar to that found in EHF 32. × 21.

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The Duration of Fertility and the Histological Changes in the Reproductive Organs after Ligation of the Vasa Efferentia in the Rat.

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[Plates 16 and 17.]

In a previous paper (White, 1932) the survival of spermatozoa in the male rat after ligation of the vasa efferentia was reported, motility being the criterion of survival. An attempt has now been made to determine the duration of fertility in animals similarly treated and to correlate motility with fertility. Data on these subjects are given in the present paper, together with certain observations on the histological changes occurring in the reproductive system after vasa-efferentia ligation.

Material and Methods.—Fifty-three males and a larger number of females were used in this work. The procedure of vasa-efferentia ligation was the same as used in previous studies and was briefly as follows: under full anæsthesia with ether, a median incision was made in the scrotum, exposing the tunica vaginalis of the testis. The testis and epididymis were then withdrawn, through an opening in this sheath, and the vasa efferentia (ductuli efferentes) dissected out of the fat lying between the testis and the head of the

epididymis. These ducts were carefully ligated in two places and severed between the ligatures. The testis and epididymis were then replaced in the sheath, which was sutured with silk, and the skin wound closed with suture clips. Aseptic technique was used throughout.

After operation the rats were returned to their cages and females added at the desired time. In the early experiments two œstrous females were placed with each male in the afternoon. If no sperm was found in the vaginæ the following morning, another female was added that afternoon, The number of animals copulating was very small, possibly owing to the females going off œstrus too quickly. For this reason in all the later tests three females, chosen at random, were placed with the male. The vaginal contents were then examined for sperm on each of the next six days. In every test there was at least one female in œstrus during the first two days, and the number of copulations was considerably increased over the early experiments.

Females were placed with the males at intervals of 3 days up to 30 days after operation. Three day intervals were chosen, since this seems to be about the least difference in sperm-survival time significant under the existing conditions. Pregnant females were isolated and the date of parturition and number of young recorded. The remaining females were left with the males for about three months to make sure that no further pregnancies, due to incomplete ligations, occurred. All males which had at some time been fertile were then killed and examined for incomplete ligations, and the testes and accessory organs sectioned to study the changes caused by the operation.

Duration of Fertility.—From the data given in Table I it becomes evident that the rat is fertile for about 21 days after ligation. When the fertility is calculated on the basis of number of litters per operated animal, the fertility is fairly low. Probably a more correct estimate is obtained when the number

Table I.—Fertility of Rats after Vasa-efferentia Ligation.

Time after operation, days.	No. of animals.	No. of copulations detected.	No. of litters.	Size of litters.
3	3	2	2	5, 1
6	4	1	1	6
9	8	4	3	8, 4, 3
12	3	2	1	4
15	9	3	2	3, 2
18	6	5	4	4, 4, 3, 1
21	7	3	1	2
24	7	0	0	—
30	6	0	0	—

of litters is considered in relation to the number of males actually copulating. The number of animals available for each of the three-day intervals is not sufficient to permit of statistical treatment. Actually, one-third of the males were still fertile at 21 days, after which there was complete sterility, no litters being obtained from any of the seven rats mated 24 days after ligation or from the six mated after 30 days. There is a bare possibility that some of the animals of the last two groups were still fertile, as no copulations were detected. Failure to detect mating may have been due to its absence or to insufficiency of sperm in the vagina.

It has already been shown that sperm survive in the vasa-efferentia ligated rat for 42 days (White, 1932). Moore's (1928) method was used for quantitatively estimating the degree of motility on removal from the epididymis. Violent activity on the part of all sperm is rated **** by this method. The designation, when only a few sperm are inactive and the majority still quite motile is ***. ** is used when about half the sperm are still somewhat motile, and * when only a very few sperm are still slightly active. When no motility, can be detected in any sperm the reading is 0. By this system, potential motility persists in sperm in the tail of the epididymis, as follows :—**** for six days, *** for 15 days, ** for 27 days, and * for 42 days after ligation. From the data on the percentage of fertile matings, complete fertility seems to persist for six days, about 80% fertility for 18 days, and 33% fertility

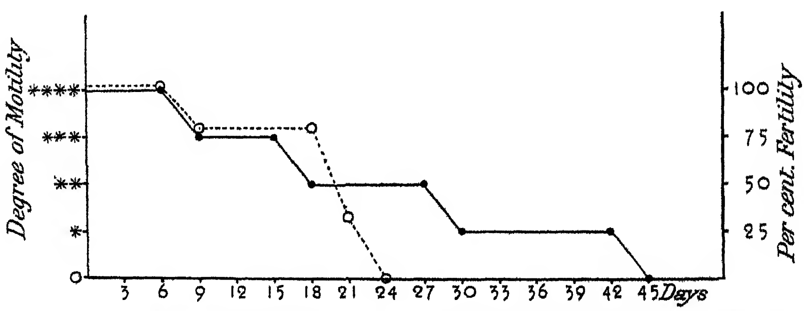

FIG. 1. ——— Degree of motility, - - - - - percentage fertility, after vasa-efferentia ligation in the rat.

for 21 days. The number of animals is small, but the duration of fertility is certainly of this order of time. The relation between degree of motility and percentage of fertile matings is shown graphically in fig. 1. Although only 75%, 50%, and 66% fertility was recorded in the individuals tested after 9, 12, and 15 days respectively, the number of animals is small, and the graph is accordingly plotted to show the 80% fertility which was found after

18 days ligation. It seems probable that the other points would approach this level if sufficient animals were used.

Although there is certainly a tendency towards the production of small litters as sterility is approached in the rat, the data on this point are not so striking as were found by Hammond and Asdell (1926) for the rabbit. The average size of the litters produced during the first nine days after ligation was 4·5, while those produced by males after nine days averaged 2·9. Both figures are below the average size of litters from normal rats of the Institute colony, which is about seven or eight.

Stone (1932) has reported extensive experiments on the effect of castration on mating in the rat. It seemed desirable to secure data on the survival of fertility in such animals, since sperm, capable of becoming motile when removed from the epididymis, can be recovered for only 21 days after removal of the testes (White, 1932), whereas they can be found for 42 days after vasa-efferentia ligation. Accordingly twelve rats were castrated by a scrotal approach and placed with females after varying intervals. None of these copulated during the first six days, and any subsequent matings were infertile.

Effect of Vasa-efferentia Ligation on the Testis.—In a previous paper it was shown that definite degeneration of the seminiferous tubules follows ligation of the vasa efferentia. This is in agreement with the earlier findings of Van Wagenen (1925), Oslund (1926) and Cunningham (1928). Moore (1932) has recently questioned these results. The fact that spermatozoa have been found (*a*) in grafts of immature testes recovered six months after transplantation and (*b*) in the testes of an adult guinea pig with congenital absence of the Wolffian duct, in which about 50% of the seminiferous tubules were in a normal state, are to him conclusive evidence that occlusion of the vasa efferentia cannot be held responsible for testis degeneration.

The conditions produced by sudden ligation of the efferent ducts of a mature testis, which is rapidly liberating spermatozoa, do not seem altogether comparable to those found in testis tissue persisting without ducts since early development. The rats used in the present fertility work have afforded additional data on this subject. The immediately apparent causes which might effect degeneration of the testis, after a procedure such as vasa-efferentia ligation, are failure of blood supply, increased internal pressure, exposure of the testis to the higher abdominal temperature and, possibly, infection. Since the spermatic arteries and veins were always carefully avoided and left undamaged, blood stasis is probably not altogether responsible for the degeneration. The post-operative condition does not resemble that of an

infarct histologically, and as shown below, the interstitial cells were functioning normally.

It is highly desirable that sperm shall not be subjected to abdominal temperatures in work dealing with their survival. Accordingly a scrotal approach to the testis and vasa efferentia was used throughout this study and no testes have been found lodged in the abdomen after operation.

The rat is very resistant to ordinary infections, but, nevertheless, sterile procedure was employed in all operations, and no animals have become infected. There are thus left pressure atrophy or some still unknown factor, as possible causes for the degeneration. The testis is certainly under considerable pressure soon after ligation. It becomes quite firm for several days, afterwards gradually decreasing in size and becoming slightly flabby. These stages have already been described. It is with the later changes that the present work is concerned.

Moore (1931) ligated the head of the epididymis in guinea-pigs so that one-third of the epididymis remained intact. Six months later he found from 60% to 80% of the seminiferous tubules normal. He concluded that degeneration had not taken place. Failure to degenerate may have been due to incomplete occlusion of some of the ducts, or to the distance from the testis at which the ligature was applied. Since it is now generally agreed that ligation of the ductus deferens does not cause appreciable changes in the testis, it is probable that the passages must be ligated immediately adjacent to the testis to cause marked degeneration. It is possible, also, that in Moore's experiments degeneration did take place and that regeneration occurred within six months. No animals were examined earlier to determine this point. It is unquestionable that if all the vasa efferentia are securely ligatured, degeneration follows in the testis of the rat. It has not been determined whether this degeneration is permanent. Oslund saw no evidences of regeneration two months after ligation. In addition to animals previously used the writer has examined the testes of three rats two and a half months after ligation, of twelve rats four months after, and of six others seven months after.

By ten weeks after ligation the degeneration is far advanced. Only a reticulum of Sertoli cells and an occasional spermatogonium are found in the seminiferous tubules, whose diameter is only about half that found in the normal rat. Likewise considerably fewer tubules are present in comparable transverse sections. The syncytia of necrotic cells, present earlier, have disappeared after ligation. However, a few tubules are still filled with debris in which calcium is beginning to be deposited. There is considerably more

interstitial tissue evident at this stage, but after rough quantitative estimates, following the method of Bascom (1925), this increase seems to be only relative and not a real hyperplasia. In a number of animals a few tubules do not degenerate. These normal tubules may be clumped in a single area, suggesting a common outlet for their products, or they may be somewhat scattered over the section. Their number, however, does not exceed about 5% of the tubules.

After four months the condition in the testis is little changed, figs. 1, 2, Plate 16. It seemed possible that regeneration might occur if the pressure in the testis, which at this time is slight, was relieved. Accordingly one testis was exposed in each of two animals four months after ligation. The fibrosed ducts were cut away as they emerged from the testis, and at the same time the intertubular fluid was drained off by puncturing the tunica. The other testis in each case acted as a control. These animals—together with six others—were examined seven months after ligation. Definite regeneration seemed to have occurred. About 15% of the tubules were normal in appearance and another 10%—although smaller in size and less compact in structure—had active germ cells, several groups of dividing cells being found in each section, fig. 3, Plate 16. The presence of an occasional syncytium of necrotic cells indicated that some of these newly active tubules were again undergoing degeneration. There was no appreciable difference between the testes which had been drained and the others; all showed some regeneration.

Although ligation of the vasa efferentia causes the appearance of castration cells in the anterior pituitary body (Van Wagenen, 1924, and unpublished observations of A. E. Severinghaus) there seems to be very slight, if any, decrease in the productions of testis hormone, as judged by the histological appearance of the accessory organs, figs. 5 and 6, Plate 16; figs 7–11, Plate 17.

Summary.

1. While motile sperm may be found for 42 days, fertility persists for only 21 days after vasa-efferentia ligation in the rat, at which time about half the sperm are motile on removal from the epididymis.

2. Extensive degeneration of the seminiferous tubules follows vasa efferentia ligation. Regeneration of about 25% of the tubules occurs within seven months after operation.

3. As judged by histological examination of the accessory reproductive organs, there is little, if any, decrease in endocrine activity of the testes following the operation.

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DESCRIPTION OF PLATES.

PLATE 16.

FIG. 1.—Testis four months after vasa-efferentia ligation, showing advanced degeneration of seminiferous epithelium and presence of intertubular fluid. × 9.

FIG. 2.—Higher power of fig. 1. × 60.

FIG. 3.—Testis seven months after vasa-efferentia ligation, showing mitotic figures and partial regeneration. × 60.

FIG. 4.—Normal testis for comparison with figs. 2 and 3. × 60.

FIG. 5.—Ductus deferens four months after castration, showing degenerative changes. × 120.

FIG. 6.—Ductus deferens four months after vasa-efferentia ligation, showing active normal condition. × 120.

PLATE 17.

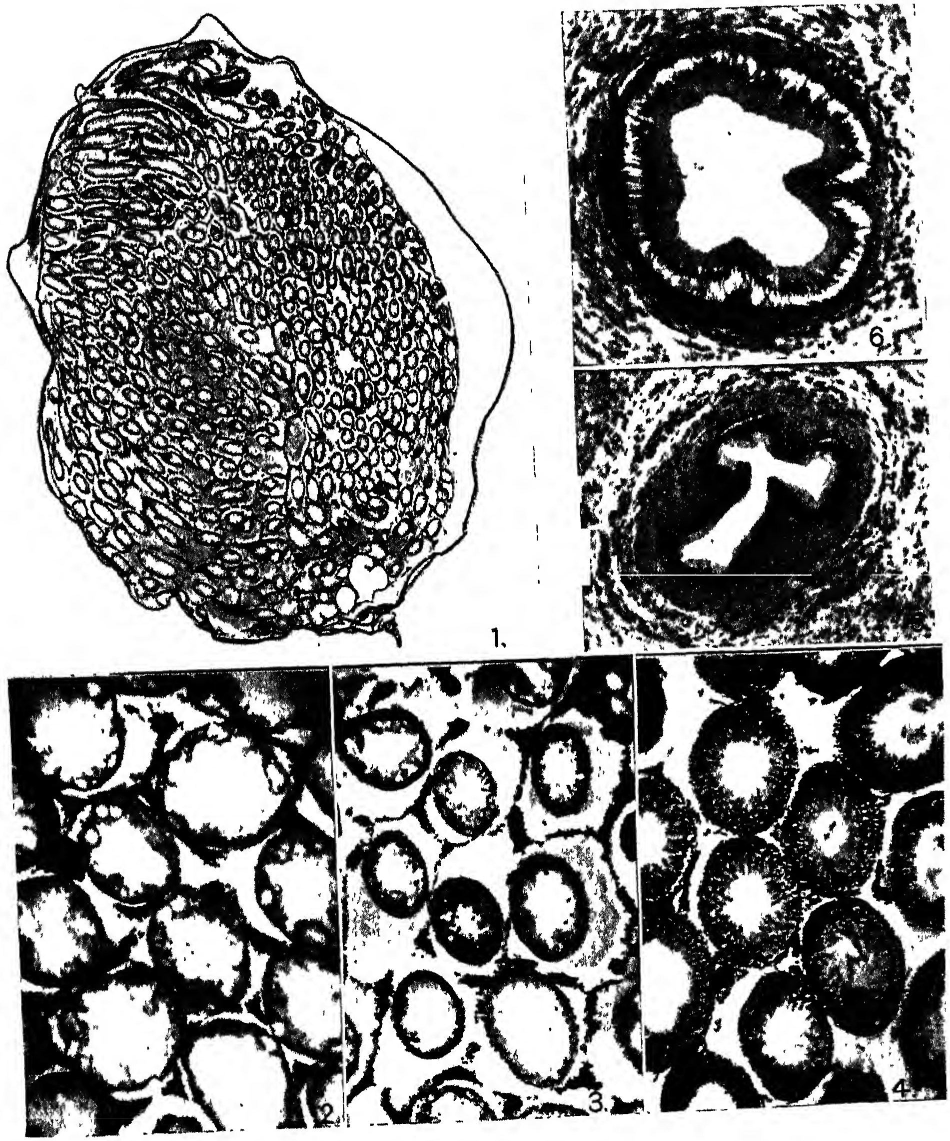
FIG. 7.—Seminal vesicle four months after vasa-efferentia ligation, showing normal condition. × 140.

FIG. 8.—Seminal vesicle four months after castration, showing degenerate epithelium and lack of secretion. × 160.

FIG. 9.—Normal epididymis (tail) showing high epithelium and presence of spermatozoa. × 215.

FIG. 10.—Epididymis (tail) four months after vasa-efferentia ligation, showing active epithelium and absence of spermatozoa. × 215.

FIG. 11.—Epididymis (tail) four months after castration, showing degenerate epithelium and absence of spermatozoa. × 215.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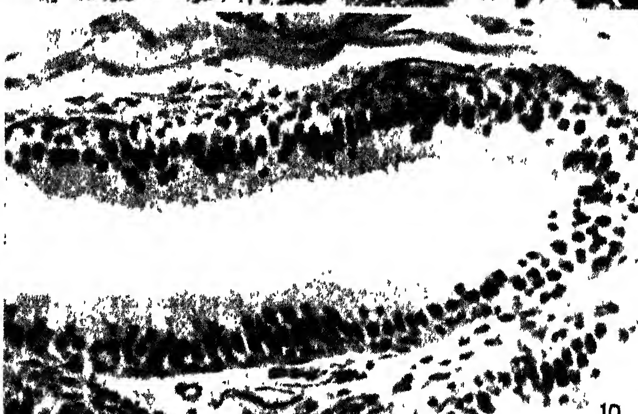

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8.

11.

INDEX TO VOL. CXIII. (B)

END OF THE ONE HUNDRED AND THIRTEENTH VOLUME (SERIES B).